C-REACTIVE PROTEIN IN PERIODONTAL DISEASE

BY

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DECLARATION

I declare that this thesis has been composed by myself and that the work described is my own, except where acknowledged in the text by reference.

SABAH ISAAC AZIZ

FIRST DEDICATION

This thesis is mainly dedicated

To

JULIET

(my wonderful and dear wife who supported me during my study), and also dedicated to my daughter(**FRANCES**) and my son (**FADIE**)

Yours

SABAH

Secondary Dedication

I would like to honor at this moment, the hard life sacrificed for me by my living mother and deceased father, and respectively I dedicate this thesis to them.

Your son

SABAH

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ABSTRACT

The aim of this study was to investigate the role of the acute phase protein (C-reactive protein) as a possible diagnostic aid in periodontal disease activity using gingival crevicular fluid (GCF), saliva and serum, and to investigate the value of measuring CRP in predicting periodontal disease activity.

Fifty-four patients with chronic periodontitis and twenty five healthy dental students (controls) from Edinburgh Dental Hospital and School were recruited for the study, and were seen at two months intervals over one year.

Clinical parameters were recorded, microbiological samples taken and biological fluids sampled at each of the six examinations over the study period. GCF, saliva and serum samples were analysed for CRP levels using a modified ELISA technique developed during the study.

The clinical, microbiological and CRP data was subjected to statistical analysis using a general statistic package.

The results confirm the presence of CRP in GCF for both the patient and control groups, and this is the first report for CRP being present in GCF.

The results indicated a significant positive correlation between attachment level change and changes in CRP levels for both short and long term.

However, within the limitations of this study, CRP levels in GCF failed to predict periodontal disease activity as

measured by attachment level loss.

It is concluded that CRP is present in GCF and that its level in GCF is significantly associated with attachment level change. The level of CRP in GCF cannot be used as a diagnostic aid in predicting periodontal disease activity over a period of two months or one year.

It is suggested that further investigation should be carried out to monitor CRP levels within a very short time period as the level rapidly increases during chronic inflammation and rapidly returns to a normal level when the inflammatory stimulus subsides.

CONTENTS

Declaration		
Dedication		ii
Acknowledgements		iv
Abstract		v
CONTENTS		vii
CHAPTER ONE: Review of Literature		
1.1	General Introduction	1
1.2	Clinical features and histological appearance of the periodontium in health and disease	8
1.2.1	The periodontium in health	8
1.2.2	Periodontal disease	9
1.2.2.1	Epidemiological studies on periodontal disease	9
1.2.2.2	Clinical and histopathological features of periodontal disease	12
1.2.2.2.1	Gingivitis	12
1.2.2.2.2	The Advanced Lesion- Chronic Periodontitis	14
1.2.2.3	Classification of chronic periodontitis	15
1.3	Aetiology and pathogenesis of periodontitis	18
1.4	Patterns of destructive periodontal disease (Periodontal disease activity)	21
1.5	Diagnosis of Periodontal disease	24
1.5.1	Clinical Methods	25
1.5.1.1	Assessment of oral hygiene	26
1.5.1.2	Assessment of the gingival inflammatory condition	27
1.5.1.2.1	Gingival indices	27
1.5.1.2.2	Bleeding indices	28
1.5.1.2.3	Gingival crevicular fluid (GCF) flow	32

1.5.1.2.4	Suppuration	32
1.5.1.3	Measurement of probing (pocket) depth and clinical attachment level	32
1.5.1.3.1	Definitions	32
1.5.1.3.2	Measurement and detection of periodontal disease activity (PDA)	33
1.5.1.3.3	Incidence of Attachment Level Changes	35
1.5.1.3.4	Errors associated with attachment level measurements	38
1.5.1.3.5	Methods of statistical analysis for the detection of attachment level changes	43
1.5.1.4	Radiographs	51
1.5.1.5	The ability of the clinical parameters to predict periodontal disease activity	51
1.5.2	Biochemical Methods of Diagnosis and Gingival Crevicular Fluid (GCF)	58
1.5.3.1	Gingival crevicular fluid; Definition, Production, Sampling methods and volume measurement	59
1.5.3.2	Constituents of GCF and disease activity	69
1.5.3	Microbiological methods	71
1.5.3.1	Microscopic Methods	71
1.5.3.2	Microscopic studies of subgingival microflora in periodontal disease and their relation to clinical parameters	74
1.5.3.3	Microscopic assessment in prediction of periodontal disease activity	78
1.5.4	Immunological methods	81
1.6	Acute phase response	83
1.6.1	Introduction	83
1.6.2	Induction and regulation of acute phase response and protein synthesis	85
1.6.3	Interleukin-6 and the acute phase response	88
1.6.4	Regulation of C-reactive protein synthesis	90

1.7	C-reactive protein	
1.7.1	Historical review	95
1.7.2	Biosynthesis of C-reactive protein	97
1.7.2.1	Hepatic origin of CRP synthesis	97
1.7.2.2	Extrahepatic origin of CRP synthesis	99
1.7.3	Secretion, metabolism and kinetics of CRP synthesis	99
1.7.4	CRP structure	102
1.7.5	Forms of CRP	104
1.7.6	Serum CRP Levels	106
1.7.6.1	Serum CRP levels in healthy subjects	107
1.7.6.2	Serum CRP levels in diseases	108
1.7.7	Binding specificities of CRP	109
1.7.7.1	Binding at the sites of inflammation	109
1.7.7.2	CRP Binding sites	111
1.7.7.3	CRP binding reactivities	112
1.7.7.3.1	Calcium and non calcium-dependent binding	112
1.7.7.3.2	Binding of CRP to Liposomes	115
1.7.7.3.3	Antibodies	115
1.7.7.3.4	Fibronectin	116
1.7.7.3.5	Chromatin (Cr) and Nuclei (Nucleic acids, Nucleosome core particles)	117
1.7.8	CRP interactions with cellular elements of immune system, and complement system	119
1.7.8.1	CRP interactions with Neutrophils	119
1.7.8.2	CRP interactions with Monocytes/Macrophages	127
1.7.8.3	CRP interactions with Lymphocytes	132
1.7.8.4	CRP interactions with Complement system	132
1.7.8.5	CRP interactions with Platelets	145
1.7.9	Biological functions of C-reactive protein	149

1.7.10 CRP and Oral Diseases 160

CHAPTER TWO

Clinical Materials and Methods: Aims, Subjects, Experimental Design, Clinical methods and data Analysis

2.1 Subjects 162

2.2 Selection of sites 166

2.3 Aims of the study 167

2.3.1 General Aims 167

2.3.2 Specific Aims 167

2.4 Experimental design 169

2.5 Clinical Methods 170

2.5.1 Plaque index (PI) 171

2.5.2 Gingival index (GI) 171

2.5.3 Bleeding on probing (BOP) 172

2.5.4 Pocket depth (PD) and Attachment level measurement 173

2.5.4.1 Development of a reproducible method for measuring attachment level changes and pocket depth (Modified stent technique) 173

2.5.4.2 Measurement of pocket depth and attachment level changes during longitudinal studies of untreated periodontal disease 176

2.5.4.3 Detection of attachment level changes 176

2.5.4.4 Classification of attachment level changes 177

2.6 Collection of samples 179

2.6.1 Collection of the saliva 179

2.6.2 Gingival crevicular fluid (GCF) collection 179

2.6.3 Subgingival plaque 180

2.6.4 Detection of subgingival microorganisms using phase-contrast microscopy (PCM) 181

2.6.5 Blood sampling 183

2.7 Data Analysis 184

2.7.1	Identification of attachment level change at each periodontal site	184
2.7.2	Classification of periodontal sites according to attachment level change in the longitudinal study	89
2.7.3	Statistical Analysis	195
CHAPTER THREE: Laboratory Materials and Methods		
3.1	Materials	203
3.1.1	Buffers	203
3.1.1.1	Coating Buffer	203
3.1.1.2	Phosphate buffered saline (PBS)	203
3.1.1.3	Washing and diluting Buffer (PBST-20)	203
3.1.1.4	Phosphate citric buffer	204
3.1.1.5	Fetal Bovine serum	204
3.1.2	Purified standard C-reactive protein and antisera for enzyme linked immunosorbant assay (ELISA)	204
3.1.2.1	Rabbit Anti-Human C-Reactive Protein (IgG Fraction of Antiserum Developed in Rabbit	204
3.1.2.2	Goat Anti-Rabbit IgG (Whole Molecule) Horse raddish peroxidase conjugate	204
3.1.2.3	Goat Anti-Human C-Reactive Protein (CRP) developed in goat	205
3.1.2.4	Substrate	205
3.1.2.5	Substrate buffer solution	205
3.1.2.6	Standard C-Reactive Protein (STD CRP)	205
3.1.3	Microtitration plates	206
3.1.4.	Multiskan Reader II	206
3.2	Methods	207
3.2.1	Calibration of Periotron 6000	207
3.2.2	Determination of GCF volumes	208
3.2.3	Elution of GCF from periopaper	212

3.3	Development of a modified ELISA technique for quantification of CRP in Serum, Saliva and gingival crevicular fluid	213
3.3.1	Sandwich ELISA methodology	213
3.3.2	Establishment of CRP sandwich ELISA	215
3.3.3	Pilot study in the quantification of C-reactive protein (CRP) in serum, saliva and GCF using double sandwich ELISA	221
3.3.3.1	Preliminary experiments to detect and quantify CRP in GCF	221
3.3.3.2	Assessment of the recovery of CRP protein from periopaper in vitro	227
3.3.3.3	Quantification of serum CRP	230
3.3.3.4	Comparison of ELISA with Laser nephelometry in CRP determination	230
3.3.3.5	Quantification of CRP in saliva	235

CHAPTER FOUR: RESULTS

4.1	Attachment level changes in both periodontal patient and healthy control groups during the longitudinal study	238
4.1.1	Incidence of attachment level change over one year determined by linear regression analysis method	238
4.1.2	Incidence of attachment level change during two months intervals between each successive visits determined by a safety threshold method	241
4.2	Clinical, microbiological and laboratory findings using the subject as an experimental unit of analysis	245
4.2.1	Clinical findings in patient and control group	247
4.2.2	Association of attachment level change with clinical parameters (PI,GI,BOP,PD), and GCF volume in the patient and control groups	250
4.2.3	Association of attachment level change with bacterial morphotypes (coccoid,other, motile rods, spirochaetes) in the patient and control groups	252

4.2.4	Laboratory findings on serum C-reactive protein and salivary CRP in the patient and control groups	256
4.2.4.1	Correlation of serum and saliva CRP levels in each subject group	258
4.2.4.2	Comparison between control and patient groups for their serum and salivary CRP levels	258
4.2.4.3	Association of serum and saliva CRP with attachment level changes in each group	261
4.3	Clinical, microbiologic and laboratory findings in the patient and control groups using the site as an experimental unit of analysis	263
4.3.1	Clinical findings (PI,GI,BOP,PD,attachment level measurement) and GCF volume for periodontal sites in patient and control groups	263
4.3.2	Microbiologic findings (coccoid cells,other , motile rods and spirochaetes) in the patient and control groups	280
4.3.3	Association of attachment level change per site with clinical and microbiological variables in each group	282
4.3.4	Laboratory findings of GCF CRP (GCF CRP concentration, GCF CRP secretion)	289
4.3.4.1	Reporting of GCF CRP results	289
4.3.4.2	GCF CRP levels for all periodontal sites in the patient and control groups	289
4.3.4.3	GCF CRP levels for each group of sites in periodontal patients during a longitudinal study.	292
4.4	Association of GCF CRP level with microbiologic findings, clinical parameters and GCF volume at each site group	295
4.4.1	Association of GCF CRP levels with subgingival bacterial morphotypes	295
4.4.2	Association of GCF CRP levels with PI,GI, BOP.	298
4.4.3	Association of GCF CRP levels with PD and GCF volume	300

4.4.4	Association of GCF CRP levels with attachment level	303
4.4.4.1	Association of the change in GCF CRP level with attachment level change for all sites in the control and patient groups	303
4.4.4.2	Association of GCF CRP levels with attachment level change at each site group in periodontal patients	312
4.5	Differences in clinical parameters, subgingival bacteria and GCF CRP levels among site groups	314
4.6	GCF CRP levels for loser and stable periodontal sites at the initial visit, at the subsequent visit of each pair of successive visits, and their differences	319
4.6.1	GCF CRP levels for all loser and all stable periodontal sites for all patients	319
4.6.1.1	The levels of GCF CRP concentration and secretion	320
4.6.1.2	Comparison of GCF CRP levels at all loser and all stable sites	323
4.6.2	GCF CRP levels for matched pairs of the most loser site and the most stable periodontal site in the patient group	327
4.6.2.1	The level of GCF CRP secretion and concentration	327
4.6.2.2	Comparison of GCF CRP levels for matched pairs of loser and stable sites	331
4.7	Predictability of GCF CRP for periodontal disease activity in periodontal patients	335
4.7.1	Predictability of GCF CRP for periodontal disease activity using all loser and stable sites	336
4.7.2	Predictability of GCF CRP for periodontal disease activity using matched pairs of loser and stable sites within each patient	341
4.8	The ability of clinical parameters, GCF CRP levels, and microbiological variables individually or collectively to discriminate between loser and stable sites.	345
4.9	The ability of clinical parameters,	352

microbiologic variables and GCF CRP levels recorded at the initial visits to predict periodontal disease activity at the subsequent visits individually or collectively.

5.1 Considerations on clinical and laboratory data 359

5.1.1 Identification of attachment level changes 359

5.1.2 Incidence of attachment level change 365

5.1.2 Reporting of GCF CRP data 367

5.2 Longitudinal study of healthy controls and chronic periodontitis patients on maintenance therapy 369

5.2.1 Clinical, microbiological and laboratory findings using the subject as an experimental unit of analysis 369

5.2.2 Clinical, microbiological and laboratory findings using the site as an experimental unit of analysis 372

5.2.2.1 Clinical findings 373

5.2.2.2 Microbiological findings 375

5.2.2.3 Laboratory findings 376

5.3 Differences in the clinical parameters, microbiological variables and GCF CRP levels among site groups 378

5.4 GCF CRP level associations with clinical parameters and microbiological variables 380

5.5 GCF CRP and periodontal disease activity 383

5.6 Predictability of clinical parameters, microbiological and GCF CRP for periodontal disease activity 392

5.7 Diagnostic Predictability of GCF CRP levels at subsequent visits for periodontal disease activity 398

5.8 Conclusion, summary and further suggestions 403

References 409

List of Tables 483

List of Figures 488

CHAPTER ONE

REVIEW of LITERATURE

1.1 General Introduction

Periodontal diseases are regarded as a range of different diseases for each of which certain individuals form a minority group and are at relatively high risk.

The epidemiological evidence for the existence of high-risk groups has shown that world-wide the prevalence of severe destructive periodontitis is of the order of only 7-15% of the adult dentate population. Successful identification of such groups, individuals or sites will undoubtedly permit scientifically valid, rational and targeted prevention and treatment of destructive periodontal disease.

Periodontitis is an inflammatory disease of the periodontium which is characterised by a progressive destruction of the tissues supporting the tooth, with loss of bone, loss of attachment and tooth mobility. Three different patterns of periodontal disease activity have been suggested, the three patterns being incorporated in three hypotheses for periodontal destruction. These are continuous paradigm, random burst and synchronous burst hypotheses.

Periodontal disease activity may manifest in different patterns at different sites and within different subjects. The disease is currently considered to be episodic with, relatively short episodes of rapid tissue destruction followed by some repair, and prolonged intervening periods of disease remission. The dental surgeon can determine that an episode of disease has occurred, rather than being able to document the present state of disease activity, but he

is unable to determine a future progression of the disease. The fundamental concepts of measuring periodontal disease emphasise the need for improved diagnostic and prognostic tests. It would be of immense value to be able to identify both those individuals at particular risk of disease as well those who have active disease at examination time. The usual clinical parameters of periodontal disease are only capable of identifying disease retrospectively, and it has emerged that there is a need for longitudinal, rather than cross sectional, studies in the search for clinical and laboratory markers of current disease activity and susceptibility. Such markers are likely to be present in the gingival crevice where the destructive process starts and where the products of the tissue destruction can be detected as well as other factors which may be involved in the destructive process. Both the clinical detection of disease progression and the study of laboratory markers including microbiological and immunological factors will in future no doubt be of great value in determining the course of the disease and influence treatment. Traditional clinical criteria of periodontal disease (e.g.pocket depth, bleeding on probing and gingival index) are inadequate in both determining an active disease site in periodontitis and the response to therapy, and in measuring the degree of susceptibility to future breakdown. The criteria are inadequate for predicting or monitoring clinical attachment loss. With the exception of attachment loss, they are of limited value in detecting individuals at high risk.

Biochemical methods in periodontal disease diagnosis have centred primarily on gingival crevicular fluid (GCF) and its constituents. Biochemical markers in GCF have been related to gingivitis and periodontitis,and most recently to periodontal disease activity. The analysis of GCF for biochemical markers of disease is envisioned to be one of a battery of adjunctive diagnostic tests available which might enable the dental surgeon to identify patients at risk of disease progression. This requires distinguishing between the extent of tissue damage and current disease activity. Tissue damage is estimated at the time of examination using clinical measures including probing depth, clinical attachment level, tooth mobility, and the level of alveolar bone support. Disease activity is evidenced by the progression of periodontal disease, determined primarily by changes in the clinical attachment level or alveolar bone loss. The biochemical analysis of GCF may offer a means of assessing the nature of the host response in the periodontal tissues, and may provide a method for the identification of patients at risk for the development of active periodontal disease.

GCF is a promising source of markers for the detection of disease activity because it contains components derived not only from plasma, from connective tissue and from host cells in the periodontium, but also components from the subgingival microbial plaque, and thus an extremely broad range of candidate markers can be investigated. Examination of components of this fluid might permit the determination

of disease markers at specific sites in the dentition. Although the identification of one or more of these markers may be shown to identify or predict disease activity, the use of standard clinical measurements of periodontal disease is not likely to be replaced in the near future. Rather, identification of markers will comprise a battery of biochemical, microbiological, and immunological tests that will allow careful, in-office monitoring of patients with periodontal disease. There is now an urgent need for the identification of accurate and sensitive markers of destructive periodontitis in order to place the diagnosis and management of disease on a more rational basis. The most appropriate biochemical markers of disease activity would be mediators or regulators of the inflammatory process, which should show dramatic quantitative changes in the presence of inflammation.

There are many potential markers which increase during the acute phase of the inflammatory process. Among these are the acute phase proteins such as serum amyloid protein, C9 and C-reactive protein (CRP).

CRP is normally present in the serum within the range of 0.05-4.0 ug/ml with a median of 0.8 ug/ml in 95% of healthy persons. This protein is increased in the serum during the acute stage of the inflammatory process up to 100-1000 fold of its normal level. It can thus be used as a marker of infection or disease activity, particularly if changes in the levels of CRP are interpreted along with other markers such as (ESR) and the peripheral white blood cell count.

CRP levels are used in this way in the diagnosis and monitoring of disease activity in cystic fibrosis, rheumatoid arthritis, rheumatic fever, myocardial infarction and malignancy.

C-reactive protein is secreted by hepatocytes under the induction of interleukin-1 (IL-1) or/and IL-6. The serum level starts to increase within 6-8 hours after the onset of acute inflammation, reaches its peak at 48-72 hours and starts to decrease by the fourth or fifth day to return to a normal level within one week of the onset, provided that there is no continuing inflammation.

C-reactive protein has been found to be deposited locally at the site of the inflammatory process, on the walls of damaged or necrotic cells, and it is thought that CRP participates directly in the inflammatory process. It has been shown also that CRP increases in the serum of patients with destructive diseases such as rheumatoid arthritis. Although the exact biological function in vivo of CRP has not yet been established, it has been suggested on the basis of in vitro studies that CRP influences the functions of phagocytes (macrophages and polymorphs) with particular influence on polymorphs.

CRP provides one of the several signals that trigger and/or regulate immune surveillance by augmentation or inhibition of various components of the immune response. This is an essential and beneficial function of CRP. CRP provides a synergistically acting signal only, a help which often facilitates homeostasis and which appears in some instances

to be an essential factor for homeostasis to occur.

It influences chemotaxis (as a chemotactic factor), opsonization of pathogens both directly and indirectly through complement, and initiates the respiratory burst activity of polymorphs and macrophages/monocytes.

CRP can be considered as an immunoregulator of acute inflammation and it can participate in a negative feedback mechanism to limit the potentially destructive effects of polymorphs on the tissues. It is therefore considered a protective factor in the non-specific immune mechanisms of the body. Furthermore, CRP activates complement through its classical pathway of activation to increase the protective function of complement, while at the same time causing a reduction of lytic complex formation from complement (C7, C8, C9) through its interruption of the alternative pathway of activation and thus preventing the ultimate damaging effect of complement on the cells. CRP also influences immunoglobulin function, particularly IgG, through the Fc receptors of both polymorphs and monocytes, as CRP is thought to stimulate the burst activity of the cells through the activation of IgG Fc receptors.

CRP has effects on platelets as it prevents their aggregation. It also inhibits prostaglandin formation and in this way minimises the damaging effect in inflammation of prostaglandins PGE_1 and PGE_2. Finally, CRP has effects on both B lymphocytes and T lymphocytes. It activates T lymphocytes and increases natural killer cell activity and cell-mediated cytotoxicity. It also activates T suppressor

cells and decreases B cell colony formation,thus decreasing IgA, IgG and IgM synthesis.

From its biological functions it seems that CRP influences most of the factors which participate in and regulate the inflammatory process both locally at a site of inflammation and systemically in the serum. The predominant function of CRP is on polymorphs which are the cells that appear first and in greatest number at the site of inflammation such as in an acute exacerbation or an increase of activity of periodontal disease which is cyclic and episodic in nature and lead to loss of attachment level and alveolar bone.

It was thus thought worthwhile to investigate the possible role of CRP in destructive periodontal disease and that is the most significant part of the laboratory studies reported in this thesis which were carried out in parallel with clinical measurements of periodontal disease. It was hoped that this clinical and laboratory investigation might indicate biochemical markers of disease activity which could have value in identifying the current status and predicting the future status of periodontal disease, and might be used in determining the most appropriate preventive and therapeutic measures in the management of destructive periodontal disease.

The current review of literature in this thesis consists of two parts. The first part covers the literature on periodontal disease, while the second part reviews the literature on CRP and its role in the acute phase response as well as its relation to oral diseases.

1.2 Clinical features and histological appearance of the periodontium in health and disease

1.2.1 The periodontium in health

The periodontium consists of the supporting tissues of the teeth which are the gingiva, periodontal ligament, cementum and alveolar bone. These supporting tissues together form a functional unit called the "attachment apparatus" (Lindhe, 1990). The term "clinically healthy periodontium" is appropriate for all sites that are disease-free, including those which have extensive attachment loss and recession as a result of previous episodes of periodontitis as occurs in successfully treated sites (Wennstrom, 1988). The periodontium, clinically, is characterised by a healthy gingiva which has a uniform pale pink colour which may vary due to the degree of keratinisation, thickness and vascularity (Ainamo and Loe, 1966).

The gingiva consists of dense connective tissue and stratified squamous epithelium (Ainamo and Loe, 1966; Selvige, 1977). It extends from the gingival margin to the mucogingival junction except in the palate (Ainamo and Talari, 1976; Ainamo et al., 1981). It is firm in texture and nonmobile. The surface may be smooth or stippled (Amino and Loe, 1966). The gingiva in health fills the interdental space between adjacent teeth (Wennstrom, 1988).

The predominant cells in a healthy periodontium include fibroblasts and mast cells,while cementoblasts, osteoblasts

and osteoclasts are also present. In addition small numbers of inflammatory cells (but not dense inflammatory infiltrates) are found primarily within and adjacent to the junctional epithelium. This inflammatory infiltrate consists of both polymorphonuclear leukocytes (PMNL) and mononuclear cells such as macrophages, lymphocytes,and plasma cells (Schroeder, 1976; Cooper et al., 1983; Thilo et al., 1986; Seymour et al., 1983). Gingival crevicular fluid may or may not be detected in healthy gingiva depending on the method of collection (Brill and Krass 1958; Loe and Holm-Pedersen, 1965; Alfano, 1974; and Cimasoni, 1983). Gingival margin bacterial plaque associated with periodontal health consists primarily of Gram positive and coccal forms and a few spirochaetes and motile forms (Theilade et al., 1966; Listgarten et al., 1975; Listgarten, 1976, and Listgarten, Lindhe and Hellden, 1978).

1.2.2 Periodontal disease

The term periodontal disease generally denotes all the diseases of the periodontium. In this thesis,the term is only used to describe chronic periodontitis.

1.2.2.1 Epidemiological studies on periodontal disease

Periodontitis is a global disease which may affect a high proportion of the population and is associated with deficient oral hygiene and increasing age. It generally

begins as a gingivitis at an early age with some individuals subsequently developing periodontitis if no treatment is given.

On the basis of epidemiological evidence it has been concluded that the world-wide prevalence of severe destructive periodontitis is of the order of only 7-15% of the adult dentate population (Johnson et al., 1988). In a cross sectional study Loe et al.(1986) reported that 8% of the population show advanced periodontal disease as determined by interproximal attachment loss and tooth mortality rates. Periodontal disease can no longer be regarded as a universally prevalent condition to which all members of the world's population are at equal risk in the absence of good oral hygiene. Periodontal disease should be regarded as a range of different diseases for each of which certain individuals are at a relatively high risk.

Many studies have shown that a relatively few individuals in each age group may have progressive periodontal disease and account for most of the sites showing severe periodontal destruction (Jenkins and Kinane, 1989). Papapanou et al.(1988) found that 23% of the subjects studied accounted for 75% of the sites found with attachment loss of more than 2mm. Longitudinal studies have shown that relatively few sites developed advanced periodontal destruction over the observation periods (Lindhe et al., 1983; Haffajee et al., 1983a,b).

Lindhe et al.(1983) found that only 3.9% and 11.6% of sites showed attachment loss more that 2mm from baseline at 3

years and 6 years respectively in 64 Swedish subjects with untreated destructive periodontitis. They also found a similar percentage (3.2%) of such sites in American subjects, followed for only 1 year. Subsequently, Lindhe et al.(1989b) reported that 12% of Japanese subjects studied over 2 years accounted for 70% of sites with periodontal deterioration of 3mm or more in attachment level. It seems therefore that relatively few subjects account for the majority of sites with active periodontal destruction over observation periods up to 6 years.

It can be concluded from these studies that periodontal disease is subject related, and only a small subset of individuals within a given population have advanced periodontal destruction. It also can be concluded that relatively few sites undergo active periodontal destruction.

1.2.2.2 Clinical and histopathological features of periodontal disease

1.2.2.2.1 Gingivitis

Gingivitis is defined as inflammation of the gingiva (Hurt et al., 1986) and is shown clinically by a change in colour (redness) and an oedematous appearance, together with an increasing tendency to bleeding on gentle probing.

Page and Schroeder (1976) described, on the experimental induction of gingivitis in dogs, a temporal sequence of the histopathological changes in the inflammatory lesions of gingivitis and periodontitis. Four different stages of the inflammatory condition were described; initial, early , established and advanced. There is no clear cut division between these stages of the inflammatory process in human spontaneous gingivitis, and all the changes may occur simultaneously, with the acute changes being superimposed on the chronic changes.

Clinical gingivitis is histopathologically separated into three stages following plaque accumulation at the gingival margin. These stages are:

a.Initial lesion:it occurs within 2 to 4 days after plaque accumulation in the dentogingival region, as an acute inflammatory reaction (Loe et al., 1965; Schroeder and Graf-deBeer, 1975). This lesion, which is clinically indistinguishable from normal, is characterised by

increased vascular permeability and the emigration of PMN from the vessels in the connective tissue around the base of the gingival sulcus into the gingival sulcus, having migrated through the junctional epithelium with disruption of the intercellular spaces. A protein-rich fluid exudate (gingival crevicular fluid) accompanies the cellular exudate in the gingival sulcus.

b.Early lesion: this follows the initial lesion after 4 to 7 days of undisturbed plaque accumulation, and may persist for 21 days or longer as clinically undetectable gingivitis (Schroeder et al., 1973; Seymour et al.,1983). It is characterised by persistence of the initial lesion and the development of an inflammatory infiltrate dominated by lymphocytes and macrophages. Migration of PMN into the junctional epithelium and gingival sulcus continues and crevicular fluid flow reaches a peak at 6 to 12 days following the onset of clinically detectable gingivitis (Lindhe et al., 1973). Early loss of perivascular collagen is seen.

c.Established lesion: within two to three weeks after plaque accumulation, there is a shift to a plasma cell domination in the inflammatory infiltrate which affects a larger gingival area, while plasma cells predominate, B lymphocytes are present in addition to the continued presence of PMN. Disruption and hyperplasia of the junctional epithelium is seen and gingival pocket lined with pocket formation (Lindhe et al., 1980; Seymour and

Greenspan, 1979). There is continued loss of perivascular collagen, and increased exudation of GCF. Gingival inflammation is evident clinically and can be severe (Seymour et al., 1983). This lesion can persist for months or years without progression (Page 1986). The initial and early lesions are associated with a primarily Gram positive,aerobic microflora in the gingival crevice. Subgingival profileration of Gram negative anaerobes and spirochaetes occur after epithelial detachment from the tooth surface (Loe et al., 1965,Theilade et al., 1966).

1.2.2.2.2 The Advanced Lesion- Chronic Periodontitis

This is chracterised by the loss of connective tissue attachment and alveolar bone. Ulceration of the pocket epithelium may be present and osteoclasts may be found at bone resorption areas. The 'advanced' lesion is synonymous with periodontitis. both destruction of the connective tissue attachment to the root surface and resorption of alveolar bone are seen (Schroeder and Lindhe, 1975; Seymour et al., 1979).

Periodontitis maintains all the features of the established lesion in addition to apical migration of the junctional epithelium and true pocketing (Slots, 1977). Clinically, it is characterised by the presence of gingival inflammation, periodontal pocketing, and loss of probing attachment while the loss of alveolar bone may be radiographically apparent.

1.2.2.3 Classification of chronic periodontitis

Periodontitis has been classified into four major forms depending on the severity pattern of the disease and the age of onset. These forms are:

a.Adult periodontitis:

This is the most common form of chronic periodontitis. It probably starts in adolescence and continues for the life of the individual, but it is first clinically significant in the mid-thirties (Page and Schroeder, 1982). It has no sex predilection. The prevalence and severity of the disease increase with age, and the progress is generally slow and continuous when evaluated by pooled data (Marshall-Day 1955, Listgarten 1986a). The presence and severity of adult periodontitis is directly associated with the presence of plaque and calculus (Marshall-Day , 1955; Listgarten, 1986a). Bone loss can be either localized or generalized, horizontal or vertical, but not of the distinct pattern seen in juvenile periodontitis. The affected subjects have normal PMN and lymphocyte function (Van Dyke et al., 1980 and 1985). The pocket microflora varies depending on the rate of periodontal destruction, disease activity, and host resistance (Newman and Saglie, 1984). The subgingival plaque is usually composed of Gram negative rods (Actinomyces israelii, A. viscosus) and spirochaetes, but without a significant population of P. gingivalis or Actinobacillus actinomycetemcomitans.

b.Juvenile periodontitis:

Clinically this form starts around puberty and is characterised by severe angular bony defects on the first permanent molars and/or maxillary incisors (Hurt et al., 1986). The lesions are often bilaterally symmetrical (Kaslick and Chasens, 1968). It is characterised by less acute clinical inflammation than would be expected based on the severity of the disease. The rate and severity of destruction are not consistent with the relatively sparse plaque and absence of severe clinical signs of inflammation (Baer, 1971). Deep periodontal pockets can be present while the gingival tissues appear healthy. Systemically,depressed PMN chemotaxis and phagocytosis are frequent findings (Van Dyke et al., 1982). The predominant subgingival organisms in this form of periodontitis are A. actinomycetemcomitans, Capnocytophaga orchracea and Prevotella intermedius (Slots ,1976). Juvenile periodontitis may be either localized or generalized. Localized juvenile periodontitis affects only permanent first molars and/or the incisors and mostly starts at puberty. In generalised juvenile periodontitis the first molars and incisors are frequently involved, but the periodontal destruction extends to other parts of the dentition. It occurs at an early age (12 to 30 years) and is characterised by rapid and severe periodontal destruction around most of the teeth (Manson and Lehner, 1974).

c.Rapidly progressive periodontitis:

This form occurs in a young adult population, and typically

in the early twenties through to the mid-thirties. It is characterised by severe gingival inflammation and rapid loss of connective tissue attachment and alveolar bone loss (Page and Schroeder, 1982; Page et al., 1983) affecting most of the teeth. A depressed PMN chemotaxis response has been found in a high percentage of affected subjects (Van Dyke et al., 1982). Bacteria associated with rapidly progressive periodontitis include pigmenting Bacteroides (P. gingivalis, Prevotella intermedius), Fusobacterium nucleatum and Wolinella recta (Moore et al., 1983) in addition to spirochaetes (Listgarten 1976).

d.Prepubertal periodontitis:

This is a rare form of periodontitis which may be either generalised or localised. The generalised form affects both primary and secondary dentitions and begins with the eruption of the deciduous teeth (Page et al., 1983). It is characterised by severe gingival inflammation, rapid bone loss, mobility and tooth loss. The patients often have PMN and/or mononuclear leukocyte defects and are subject to other infections such as otitis media and respiratory tract infections (Suzuki, 1988). Localized prepubertal periodontitis affects some of the primary teeth only and is less aggressive, showing either PMN or mononuclear leukocyte defects.

1.3 Aetiology and pathogenesis of periodontitis

Pathogenic mechanisms:

The exact mechanisms involved in the pathogenesis of periodontitis have not been established, but the importance of bacteria is beyond doubt.

Chronic inflammation is the hallmark of periodontal disease and the accumulation of bacterial plaque has been established as responsible for the initiation and maintenance of the inflammatory process (Theilade et al., 1966; Axelsson and Lindhe, 1978). Loe et al.(1965) were the first who confirmed that plaque accumulation causes gingival inflammation. The possible means by which bacteria and their products cause periodontal damage have been explained already by Page and Schroeder (1982) and well explained and reviewed by Southam and Soames (1993). The induction and subsequent progression of the disease result from the diffusion of bacterial products through the junctional and, later, pocket epithelium into the underlying tissues, the mechanisms actually involved in the induction and progression of the destructive, chronic inflammatory lesions are unclear.

The microbial flora contains organisms which could elaborate many potentially pathogenic substances. These substances are capable of causing direct cellular damage (e.g.ammonia, hydrogen sulphide), enzymes which are capable of causing direct damage to the intercellular matrix (e.g. collagenase, non-specific proteases and hyaluronidase), as well as substances that could induce indirect tissue damage

by activating of destructive aspects of the host's own inflammatory and immune response (e.g. endotoxins, peptidoglycans).

Currently, the interactions of bacterial products with the host immune response are thought to be major importance, and numerous potential mechanisms have been suggested by which inappropriate activation of host defence systems could damage periodontal tissues.

Activation of humoral immunity leads to the differentiation of plasma cells and production of immunoglobulins, which in turn could be involved in the inflammatory response via complement activation and antibody-mediated hypersensitivity reactions. Microbial products also can cause complement activation through alternative pathway. The activation of complement results in biologically active substances which can induce vascular permeability and are chemotactic for PMN. Activation products have been identified in crevicular fluid and probably enhance the PMN migration seen in periodontal disease. Other chemotactic factors are also present and include substances produced by plaque organisms and released by injured tissues. Although PMN have a significant role in the local defence of the dentino gingival unit, they may damage periodontal tissues by mechanical disruption, release of lysosomal enzymes and specific collagenase, and the generation of eicosanoids particularly prostaglandin E_2.

The presence of antibodies to oral bacteria and the diffusion of plaque antigen into the tissues also provides

the necessary factors for immune complex formation, although no equivalent evidence for their participation in periodontal disease has been established. Immune-complex-induced injury, mediated through type III hypersensitivity, is associated with complement activation, the accumulation of PMN to release lysosomal enzymes and other factors including interleukin-1, collagenase, and prostaglandins which could be involved in connective tissue degradation and stimulation of osteoclastic activity. Cell-mediated immunity to oral bacteria is active in gingivitis and periodontitis and type IV delayed hypersensitivity could contribute to tissue injury.

The potentially damaging effects of activation of T cells by plaque antigens could be mediated by direct cytotoxic T-cell activity or through release of lymphokines affecting macrophage function, attracting them into an area of inflammation and increasing their biological activity. The increased release of cytokines, enzymes and other inflammatory mediators from lymphokine-activated macrophages is a potential mechanism which could lead to damage of periodontal tissues. Osteoclast activating factor produced by T-cells could also cause bone destruction.

It can be concluded that all the mechanisms involved in the immunopathogenesis of chronic inflammation might be involved in periodontal disease. The early lesion of experimental gingivitis has features characteristic of delayed hypersensitivity reaction, suggesting that cell-mediated immunity is involved, while the established and

advanced lesions are dominated by plasma cells suggesting that these are predominantly B-cell lesions.

1.4 Patterns of destructive periodontal disease (Periodontal disease activity)

Periodontitis is a progressively destructive change leading to loss of alveolar bone and periodontal ligament (Hurt et al., 1986). The natural history of periodontitis is marked by periods of active destruction and relative quiescence, even though the periodontal tissue remain chronically inflamed throughout (Goodson et al., 1982).

Periodontal disease activity is episodic. The duration and frequency of the periods of tissue destruction are unknown. Periodontal disease activity refers specifically to the stage of the disease associated with loss of alveolar bone and connective tissue attachment (Listgarten, 1986a), and occurs at different rates in different sites and in different patients. Different rates of disease progression have also been reported in different age groups of both untreated patients and treated patients including those on maintenance therapy. A high rate of disease progression seems to occur at a few sites in the mouth only and in a relatively small proportion of the population. Destruction of the periodontal supporting tissues, as reported by disease activity, has been explained in three models by Socransky et al.(1984). These are the continuous paradigm hypothesis, the random burst theory, and the asynchronous

multiple burst hypothesis.

The continuous paradigm (continuous disease) hypothesis implies slow, constant, and progressive tissue destruction. In this model periodontal disease has been thought to slowly and continuously progress until tooth loss occurs. This model has been supported by both cross-sectional studies (Loe et al., 1978) and longitudinal studies of sites not responsive to therapy (Badersten et al., 1985a). This hypothesis has been refuted by Socransky et al.1984. The random burst hypothesis proposes that short periods of destruction are followed by periods without destruction, occurring randomly with respect to time and at random sites within an individual (Socransky et al., 1984). Some sites show no activity, while others show one or several bursts of activity with time. The time of onset and cumulative extent of destruction vary from site to site. Certain sites within a patient's mouth may demonstrate an active burst of destructive disease which could last from a few days to a few months before going into a period of remission. Sites showing destructive periodontal activity may subsequently never undergo destructive activity again or may be subject to one or more bursts of activity at a later time. These bursts appear to occur randomly at periodontal sites throughout the mouth. Other sites may be free of destructive periodontal disease throughout the life of the individual. It is suggested that in this model there may be relatively short periods in an individual's life in which many sites undergo periodontal destruction followed by

periods of extended remission.

The asynchronous multiple burst hypothesis proposes that destruction occurs during a defined period of life and the disease then goes into remission (Socransky et al., 1984). In this model,many sites may show bursts of activity over a limited period of time, followed by an indefinite period of inactivity. The major amount of periodontal destruction occurs over a period of a few years. In this model the bursts of destructive periodontal disease activity occur with a higher frequency during certain periods of an individual's life. Several sites demonstrate bursts of destructive periodontal disease which take place synchronously. These sites show repeated bursts of activity over a definite period of time, followed by prolonged periods of inactivity. Occasional bursts may infrequently occur at certain sites at later time periods. Other sites remain free of destructive periodontal disease at all times. The difference from the random burst theory is that the majority of destructive disease activity takes place over a few years of an individual's life. Many studies support all three temporal patterns of periodontal destruction occurring and it is possible that the disease may manifest in different patterns in different individuals and sites, depending on the type of periodontal disease present (Haffajee et al., 1988).

1.5 Diagnosis of Periodontal Disease

Consequent to current concepts of the mechanisms involved in periodontal disease, diagnostic methods can be divided into those parameters which measure the lesion or defect produced by the disease (e.g.bone loss), and those which measure characteristics of the disease process (e.g. gingival inflammation).

Periodontal diseases are inflammatory in nature and visual signs of inflammation such as redness, swelling, gingival bleeding or suppuration are routine measures by which inflammatory lesions within the periodontium can be detected. Periodontal diseases are also infections and the microbial assessment of plaque may be also useful in the diagnosis of periodontal disease. The various types of destructive periodontal disease produce anatomical changes which can usually be documented. These include probing pocket depth, gingival recession, probing attachment level, tooth mobility, and radiographically determined bony changes.

The most important measure in the diagnosis of periodontal disease involves the assessment of its activity, and this can only be achieved clinically by measuring the attachment loss. Disease progression can be assessed by longitudinal documentation of probing attachment level and/or radiographic interproximal bone height.

The diagnosis of periodontal disease includes the following methods:

1.5.1 Clinical Methods

The clinical diagnosis of periodontal disease is based on using certain clinical parameters or indices which may clinically differentiate between healthy tissues, gingival inflammation, and destructive periodontal disease. These diagnostic methods are based on visual examination, use of instruments such as the periodontal probe for measuring the pocket depth and attachment level changes, as well as radiographic examination for detecting changes in the alveolar bone. Measurement of gingival crevicular fluid flow rates may also be a useful method for diagnosis.

The characteristics of reliable diagnostic methods are usually expressed as sensitivity, specificity, simplicity and diagnostic accuracy. Sensitivity is the ability of a test to detect disease progression if it is truly there. A test with high sensitivity will give relatively few false negative results. Specificity is the ability of a test to exclude disease progression if it truly is not there, and a test with high specificity will have relatively few false positive results. The ideal index or clinical parameter to be considered a diagnostic test, should have a reliable predictive value and be acceptable to clinical design and statistical analysis.

The methods used for the clinical detection of disease progression and analytic methods used to detect changes in data over time will influence the apparent prevalence of periodontal disease activity even in the same population.

The clinical methods used in the assessment or diagnosis of periodontal disease are:

1.5.1.1 Assessment of oral hygiene

Various plaque indices which can measure plaque accumulation on the surfaces of the teeth, have been used for the assessment of oral hygiene. These indices measure the area and/or thickness of plaque or material covering the tooth surface.

Indices used are the oral hygiene index (OHI) consisting of the debris and calculus indices (Greene and Vermillion, 1960) and the modified OHI (Greene and Vermillion, 1964) which assesses the tooth area covered by soft debris. Other indices include the plaque index (PI) by Silness and Loe (1964) and Loe (1967), which measures the thickness of the plaque at the gingival margin. PI (Silness and Loe 1964) is widely used today in the assessment of oral hygiene and scores plaque accumulation on a scale from 0 to 3 as follows:

Score 0 = tooth surface is clean.

Score 1 = tooth surface looks clean but plaque can be removed from the gingival third with a sharp explorer.

Score 2 = visible plaque on the tooth surface.

Score 3 = abundant plaque on the tooth surface.

The PI can give a separate recording for each of the 4 smooth surfaces of a tooth, and can be used to score

individual sites (surfaces) as a single site score, teeth as averages of four sites per tooth, and individuals or groups of teeth. The mean of the total scores for the individuals or group of teeth is generally used for the assessment. The plaque index can assess the very subtle changes in plaque accumulation (Lindhe, 1990), and can be used to study correlations between plaque and gingivitis in periodontal research. It is also useful in the assessment of oral hygiene particularly during the maintenance phase of periodontal therapy.

1.5.1.2 Assessment of the gingival inflammatory condition

The clinical signs of inflammation, including redness, swelling (oedema), and bleeding, are the primary indicators of gingival inflammation and have been incorporated in gingival and bleeding indices.

1.5.1.2.1 Gingival indices

The gingival index (GI), developed by Loe and Silness (1963) and fully described by Loe (1967), is widely used. Other types of gingival indices are the PMA index (Schour and Massler, 1947), and the modified gingival index (MGI) (Lobene et al, 1986). Both GI and MGI have been found to be significantly correlated with each other (Lobene et al., 1989).

1.5.1.2.2 Bleeding indices

These have been used in the assessment of gingival inflammation, examples being the sulcus bleeding index (SBI) (Muhlemann and Son, 1971), the bleeding index (BI) using dichotomous scoring of bleeding on probing after 10 seconds (Ainamo and Bay, 1975), and bleeding on probing (WHO, Technical report 1979; Van der Velden, 1978,1979).

The GI scores both the severity and location of the inflammation. Inflammation is scored on a scale from 0-3 and the severity of gingivitis can be assessed separately at the 4 smooth surfaces of the tooth. Whenever the gingival margin appears inflamed, it is massaged with the side of a periodontal probe, If this massage does not result in bleeding the unit score is 1; if bleeding occurs, the score is given as 2, while ulceration and "spontaneous" bleeding scores 3.

The GI was modified by Loe (1967), with regard to the manner in which bleeding is demonstrated. Originally, a score 2 was given to gingival bleeding occurring after "pressure" (Loe and Silness, 1963), but this was subsequently changed to bleeding after "probing" by Loe in 1967. So the presence or absence of bleeding is now determined after running a blunt probe along the soft tissue wall of the entrance of the gingival crevice. GI can be used to measure the gingival inflammation for specific sites, teeth, groups of teeth or individuals in a similar way to PI. A high GI has been found to be associated with increase in volume of gingival crevicular fluid (Egelberg,

1964), but both have low predictive values for periodontal disease activity (Haffajee et al., 1983b). Pressure sensitive probes have been used for the measurement of bleeding (van der Velden and De Vries, 1978) to give more accurate determinations. Gingival bleeding has been found generally to be an earlier and more sensitive sign of inflammation than other clinical signs such as redness (Muhlemann and Son, 1971), although visually inflamed sites do not always bleed (Greensten, 1984).

Gingival bleeding has been shown to be of high sensitivity but low specificity when used as a clinical parameter for predicting disease activity as measured by a statistically significant probing attachment loss (Haffajee et al., 1983b; Badersten et al., 1985c).

Van der Velden et al.(1985) found that patients susceptible to periodontal breakdown had more bleeding, and less plaque than those not showing periodontal breakdown. They hypothesized that a high value for the ratio between bleeding and plaque may act as a prognostic indicator for periodontal breakdown. Abbas et al.(1986a) found that individuals with a high bleeding to plaque ratio developed significantly more clinical inflammation in terms of bleeding and swelling of the gingiva than individuals with a low bleeding/plaque ratio. They suggest that the bleeding/plaque ratio of an individual may be regarded as a prognostic indicator for the severity of gingivitis in terms of bleeding and swelling of the gingiva.

Gingival bleeding was found to be associated with

significantly increased percentages of both spirochaetes and other motile forms (Armitage et al., 1982), and these morphotypes have been linked with periodontally diseased sites (Listgarten and Hellden 1978; Listgarten and Levin 1981; Evian et al., 1982). Gingival bleeding was also related to an increased flow of gingival crevicular fluid (Hancock et al., 1979), which has been found to be positively associated with the presence of gingival inflammation (Cimasoni, 1974).

Bleeding on probing (BOP):

It was introduced by Ainamo and Bay in 1975, and has been used in the development of clinical indices for the assessment of periodontal health and disease (Loe and Silness, 1963; Ainamo and Bay, 1975; Muhlemann and Son, 1971). A pressure sensitive probe with a standard force is the most appropriate instrument for the assessment of bleeding on probing. BOP for pocket depth has been found to be associated with gingival inflammation and may be more appropriate than GI for identifying inflammation located deeper within the periodontium as at sites affected with periodontitis (Alfano et al., 1976). BOP is interpreted as indicating the presence of inflammation in the periodontal tissues, while the absence of BOP indicates the absence of inflammation (Lang et al.,1991).

A disadvantage of indices incorporating the BOP component is that they can not distinguish between gingival inflammation and inflammation at the base of a pocket in periodontitis. So in periodontitis, it is better to use

separate indices, one for the assessment of visual changes of the gingival tissue and one for assessing BOP.

Badersten et al.(1985c) found that the predictability of BOP for periodontal breakdown never exceeded 30%,and this was confirmed by Lang et al.(1986). Recent clinical trials of the diagnostic value of BOP have generally showed that BOP has modest sensitivity and specificity, and rather low positive but high negative predictive values (Badersten et al., 1985c; Lang et al., 1986; Claffey and Shanley, 1985; Lang et al.,1991).

Cessation of gingival bleeding has been found to be associated with pocket depth reduction and gain of attachment loss (Badersten et al,.1984c). It has been found that continuously bleeding sites on probing over a two years period were more likely to develop 2mm or greater of attachment loss than sites not showing continuous bleeding although the ability of bleeding to predict this loss never exceeded 30% (Badersten et al., 1985c,Lang et al.1986). Lang et al.1986 in their study evaluating the prognostic value of BOP in identifying sites at risk for periodontal destruction during maintenance therapy, found that pockets with probing pocket depth greater or equal to 5mm had a significantly higher incidence of BOP. Patients with 16% or more BOP sites had a higher chance of attachment loss equal to or greater than 2mm.

Sensitivity and predictability calculations have shown that BOP is a limited but yet useful prognostic indicator in clinical diagnosis for patients during periodontal

maintenance phase.

1.5.1.2.3 Gingival crevicular fluid (GCF) flow

It has been used in the assessment of gingival inflammation in many studies, and a positive correlation has been found between GCF flow and the severity of gingival inflammation (Egelberg, 1966; Loe and Hom-Pederson, 1965).

1.5.1.2.4 Suppuration

It has been found that suppuration from the periodontal pocket has been related to increased gingival inflammation in sites with advanced periodontitis (Bouwsma et al., 1988).

1.5.1.3 Measurement of probing pocket depth and clinical attachment level

1.5.1.3.1 Definitions

Probing Depth:defined as the distance from the gingival margin to the location of the periodontal probe tip. This term is preferred to the term "probing pocket depth".

Clinical Attachment Level: the relative probing depth corresponding to the distance from the cemento-enamel junction (CEJ) to the location of the periodontal probe tip. This term is preferred to the term "probing attachment level".

Relative Attachment Level: the distance from a fixed

reference point on the tooth surface or a stent to the location of the periodontal probe tip.

1.5.1.3.2 Measurement and detection of periodontal disease activity (PDA)

Currently, there is no known practical clinical test for detecting deteriorating periodontal sites.

Probing depth and clinical attachment level are both used to assess the amount of periodontal destruction and periodontal disease activity and may be supplemented by radiographic examination for the presence of alveolar bone loss. The probing depth and clinical attachment level measurements depend on the identification of the most apical depth of the periodontal probe tip in the gingival crevice.

The "gold standard" for measurement of periodontal disease activity is the loss in attachment level measured at a particular periodontal site (Goodson, 1986). Active disease has occurred if there is a statistically significant loss in the clinical attachment level.

Due to numerous sources of error inherent in probing measurements, progression of periodontitis has been defined as a loss of clinical attachment of a certain magnitude over time. This magnitude has varied from > or equal to a range from 1-3mm. A loss of greater than 2 mm must occur before a reliable determination can be made that disease activity has occurred at the site (Haffajee et al.1983a,b).

Many periodontologists have used either > or equal to 2 mm (Muller et.al, 1987;Harley et al., 1987) or > or equal to 3 mm (Lindhe et al., 1983; Haffajee et al., 1983a) loss in clinical attachment level to determine whether or not a real change has taken place. The lesser threshold of attachment loss being used, the greater the frequency of deteriorating sites will be recorded (Lindhe et al., 1983). The greater the critical value chosen, the smaller is the chance of obtaining false positive results. On the other hand, the proportion of false negative results increases. The diagnosis of periodontal disease activity becomes more specific when stricter criteria are used, but this is at the expense of sensitivity. So small increments in attachment loss may occur which can not be detected because of limitations in the current methods of measurements (Ranney et al.,1987).

Determination of probing pocket depths and clinical attachment levels remain an integral part of periodontal diagnosis and determining past disease activity. Determination of disease activity should include sequential measurements of relative or clinical attachment levels. The longitudinal monitoring of attachment levels is a reliable method for determining the stability of the periodontal supporting tissues. The periodontal probe is used primarily to measure probing depth and attachment levels. These measurements estimate two important consequences of periodontitis and represent an historical record of past periodontal disease activity (Listgarten, 1972).

A disadvantage of probing measurements is that they only detect past changes in clinical attachment levels and such changes can only be detected once they have occurred.

1.5.1.3.3 Incidence of Attachment Level Changes

Frequency, magnitude, and rate of attachment loss:

Longitudinal studies of untreated periodontal patients have indicated that attachment loss occurs at small number of sites in relatively few subjects (Haffajee et al., 1983a ;Goodson et al., 1982; Lindhe et al., 1983; Harley and Watts 1987; Muller and Flores 1987; Okamoto et al., 1988; Papapanou et al., 1989). It has been found that subjects with many affected sites exhibited more new attachment loss than subjects with few affected sites (Haffajee et al., 1988). Older subjects seemed to be more at risk of disease activity than younger subjects (Haffajee et al., 1988; Papapanou et al., 1989).

The number of sites with attachment loss occurring during a given time period has been reported to be relatively low. For instance, Goodson et al.(1982) found that sequential attachment level measurements made at monthly intervals for a year when analyzed by regression analysis (at $P<0.01$), demonstrated significant increase in probeable attachment level in 5.7%, while 11.5% of all sites monitored in 22 subjects became significantly shallower.

Haffajee et al.(1983a) when using regression analysis found that 5.1% of sites showed significant loss of attachment

and 2.3% of sites showed significant attachment gain (at P< 0.01) over 1 year.

Lindhe et al.(1983) in their longitudinal studies over 1-6 years on patients with untreated periodontitis found that 3.9% of sites showed attachment loss of more than 2mm over the first three years in a Swedish group. During the 6 years period, 11.6% of sites showed loss of attachment >2mm, 37% at 2mm, while 0.2% of sites showed gain of >2mm in attachment level. In an American group, 3.5% of sites showed >2mm of additional attachment loss over one year, while 4.3% of sites showed a gain of >2mm. They found that 1.9% of sites per year exhibited attachment loss of >2mm in the 6-year study in untreated Swedish subjects with moderate periodontitis. With a less stringent criterion of periodontal breakdown, an observed attachment loss of equal to or > 2mm was found in 6.2% of Swedish subjects and in 9.1% of American subjects.

Badersten et al.(1985b) identified the sites with probing attachment loss in patients following initial nonsurgical therapy using analysis of regression (P< 0.05). They found that 5% of sites lost attachment level of >1.5mm and 10% of sites became deeper by >1mm over 24 months with 3 months intervals between examinations.

Lang et al. (1986) found that only 3.1% of sites exhibited attachment loss of 2mm or more over one year in a 2-year study in patients receiving maintenance therapy.

Badersten et al.(1987) in their studies over 2 years with subjects examined at 3 month intervals, used regression and

end-point analysis, found 12% of sites became deeper and 4% became shallower in their probing attachment level at equal or > 1.5mm threshold.

Harley et al.(1987) found that only 6 sites (0.6%) showed loss of clinical attachment of >2mm over 12 weeks.

Lindhe et al.(1989a) monitored longitudinal changes in periodontal disease in untreated subjects over 2 years at 12 months interval. They demonstrated that these subjects did not generally undergo marked additional deterioration of their periodontal conditions, and only a small number of subjects (in most age groups) showed significant amounts of additional attachment loss.

Lindhe et al.(1989b) found also that 70% of loser sites at equal to or > 3mm, occurred in 12% of the subjects who showed loser sites during both the first and second years. Most of the loser sites were at surfaces with initially advanced loss of attachment, and were most frequently detected in older subjects. They found only 0.7% of the sites they studied had lost attachment of >2 mm from base line to year 2, and 0.35% of sites were detected as loser sites in the first 12 months.

Jenkins et al.(1988) investigated the use of certain clinical parameters (pocket depth, PI and gingival redness) and microbiological criteria to predict periodontal breakdown (clinical attachment loss) during a 1-year period examined at 2-months interval in 11 subjects with untreated advanced periodontitis. Using regression analysis ($P<0.05$), they found that 1.9% of sites demonstrated loss of

attachment of more than 2mm over the year, 9.8% of sites showed attachment loss of equal to or >2mm, while 12% of sites showed loss of attachment of more than 1mm. Only 6% of sites studied showed a gain in attachment level.

Grbic et al.(1991) using a cut off point of equal to or > 2mm, found that 1% of the patients studied demonstrated clinical attachment loss at one site only and 21% for two sites over 6 months.

Haffajee et al.(1991) investigated the association of baseline clinical parameters of periodontal disease with disease progression represented by further attachment loss in untreated periodontal patients in the following year. They found that only 27.3% of subjects exhibited additional attachment loss of 3mm or more at 1 or more sites after 1 year. When they used a criterion of 2mm or more as a cut off point, 73% of subjects showed additional attachment loss.

Zappa et al.(1990) found that 5.0% of sites showed probing attachment loss of 2mm or more during the 10-months observation period of patients with untreated periodontitis.

1.5.1.3.4 Errors associated with attachment level measurements

1.Errors due to the probe:

These include thickness (diameter) of the probe tip, location, angulation, and the degree of penetration which

is probably due to uncontrolled probing forces (Listgarten, 1972). There are also errors in the graduation and placement of the probe (Van der Velden, 1978). Variation in the probing force and size (diameter) of the probe (Van der Velden (1979) can influence the penetration of the probe through the junctional epithelium and gingival tissue.

Attempts have been carried out to reduce measurement error by using a conventional periodontal probe to measure probing attachment level with and without an occlusal stent (Hafajjee et al., 1983b; Badersten et al., 1984d; Isidor et al., 1984; Clark et al., 1987; Best et al., 1990). Pressure-controlled probes have been introduced to control probing force (van der Velden and de Vries, 1978). An automated sensitive pressure probe has been introduced with automated detection of the CEJ (Jeffecot et al., 1986). Gibbs et al.(1988) developed a Florida probe, which is an electronic pressure sensitive probe capable of measuring probing depths to the nearest 0.2mm.

2.Errors in precision of the measurement:

The precision in the measurement may be due to examiner him/her self or the type of probe used (Jeffecot et al., 1986).

3.Errors related to the identification of the gingival margin location, and the texture of the gingival tissue:

Transient fluctuations in gingival margin location may produce errors in pocket depth measurements. Probe penetration may be influenced by the inflammatory status of the gingiva and deeper penetration of the probe is obtained

in areas with increased inflammation (Armitage, Svanberg and Loe, 1977). Changes in the location of the gingival margin due to inflammation or treatment will necessarily influence relative probing depth or probing depth but not necessarily influence the measurement of attachment level.

4.Errors associated with the fixed reference point:

Relatively stable landmarks which may be used for attachment level measurements include the CEJ, occlusal surfaces, restorations, markings bonded to tooth surfaces and stents , and all may influence the reproducibility of the measurements (Badersten,Nilveus and Egelberg,1984d). Occlusal stents have been used to reduce the errors associated with location of the CEJ (Isidor, Karring and Attstrom, 1984; Badersten et al., 1984d, Clark et al., 1987; Magnusson et al. 1988). On the other hand, Jeffecot et al.(1986) developed a controlled force probe which electronically detected the CEJ and automatically recorded probing depth and attachment level. Magnusson et al.(1988) and Birek et al.(1987) employed a pressure-sensitive device which electronically measured and recorded probing attachment level using a stent or occlusal and incisal surface as a fixed reference point.

Studies on the reproducibilty of probing attachment level measurements have shown that the difference between duplicate readings may occasionally be as much as 2 mm or more. However, the vast majority of recordings can be reproduced with a difference of not more than 1 mm (Glavind and Loe 1967; Badersten et al., 1981; Cereck et al,.1983;

Isidor et al.,1984; Badersten et al.,1984d; Harley et al.1987).

The results of previous studies on the reproducibility of attachment level measurements using onlay techniques are shown in Table 1.1.

Isidor et al.(1984) used flexible splints to produce readily identifiable reference points, and found a compatibility of 60% for pocket depth and attachment level measurements. A deviation of 1mm or less was found for 95% of the surfaces. Badersten et al.(1984d) using an onlay reference (occlusal stent) found that 90% of probing attachment level measurements were reproducible within +_1.0 mm difference. Measurement using onlay margins for reference point demonstrated somewhat less variability than the use of CEJ as a reference point. Harley et al.(1987) using a stent technique and pressure controlled probe, showed that 91% of probing depths and attachment levels remained within +_1mm. Clark et al.(1987) using a stent technique, concluded that measurements using the stent are more reliable than using the CEJ.

The level of reproducibility varied notably between patients, and between PD, tooth surfaces and types (Glavind and Loe, 1967; Loe et al., 1978; van der Velden and de Veries 1980; Goodson et al., 1982; Badersten et al.1984d; Watts 1987).

Table 1.1: The reproducibility of attachment level measurements using onlay methods.

Reference	**Reproducibility of attachment level measurements within +_1mm**
Isidor et al.(1984)	95%
Badersten et al.(1984d)	90%
Harley et al. (1987)	91%

1.5.1.3.5 Methods of statistical analysis for the detection of attachment level changes

The introduction of reliable and reproducible methods that can detect a change in attachment level which safely exceeds measurement errors is of crucial value for the measurement of periodontal disease activity, diagnosis and treatment.

Specific amounts of probing attachment loss have been used to define thresholds for periodontal disease activity (Haffajee et al., 1983a; Goodson et al., 1982; Lindhe et al., 1983; Harley et al., 1987; Muller et al., 1987). These criteria were established to reduce the possibility of false positive results due to probing errors.

To determine that loss of attachment has occurred at the clinical level, it is important to take repeated measurements of clinical attachment level over a separate time. The methods used for the clinical detection of disease progression and the analytic methods used to detect changes in the data over time can influence the apparent prevalence of periodontal disease activity. Thus the use of different analytic methods or clinical assessments may result in strikingly different prevalences of active disease even in the same population being reported. The interpretion of probing measurements must be carefully handled.

Measurement error:

Since only a small proportion of sites are defined as

experiencing disease activity in unit time, several authors have suggested that a high percentage of these could be due to measurement errors (Imrey 1986; Clark et al., 1987; Cohen and Ralls 1988, Blomqvist 1987). Differences between duplicate attachment level measurements exceeding 3 times the standard deviation have been used as the criterion for disease activity, on the assumption that recorded differences were normally distributed (Haffajee et al., 1988; Haffajee et al., 1983a,b). However, Janssen et al.(1987) warned that a normal distribution of errors associated with duplicate measurement has never been proven and other investigator have reported that differences between attachment level measurements are not normally distributed (Badersten et al., 1984d; Glavind and Loe 1967; Gonsollely and Best, 1988). Acceptance of the premise that a normal distribution of errors is true underestimates probing errors of reported proportions of sites that show 2mm gain or loss of attachment and the writers cautioned that data derived from sites using this criterion weakly corroborate the burst hypothesis.

Gunsolley and Best (1988) reported that only 70% of a measured 3mm alterations might represent actual change and 30% could be accounted for by measurement error. Any measurement alteration should include both measurement error and actual change, and careful consideration should be given to the technical problems associated with detecting a small number of sites manifesting a variable change in clinical attachment.

Statistical Methods:

Various statistical methods have been used to test for the significance of recorded changes in the attachment level. These include linear regression analysis, running medians, tolerance method, end-point analysis and other methods which based on the specificity and sensitivity of attachment level measurement to reduce both Type I and/or Type II error rate as well as the false positive rate.

IIn general these statistical analyses have been used to define and reduce the measurement errors, to detect minor attachment level change as well as to test the reproducibility of attachment level measurement. Each method has strength and weakness.

Linear regression analysis method: in this method (Goodson et al., 1982; Badersten et al., 1985b; Haffajee et al., 1983a), the slope and the intercept of a regression line are computed from a linear least squares fit of attachment level measurements as a function of time, and the slope is tested for a significant departure from zero while a threshold of projected attachment level change per unit time also has to be exceeded. This method is better suited to assessing smooth alterations than bursts of disease activity (Aeppli and Pihlstrom, 1989). Calculation of the slope of the regression line determines the rate of loss over time. Determination of the probability level for the individual slope considers the variability of the individual data points about the regression line,i.e. it includes consideration of the level of reproducibility of

the measurements at the specific site.

From a clinical point of view, the decision to classify a given site as a site with probing attachment loss may need to be based upon the magnitude of the projected difference over the observation interval (for example change over a year) in conjunction with the probability level.

Tolerance method: this designates locations manifesting disease activity if they surpass each one of three thresholds based on measurement error. These include 2 standard deviations (SD) in the population, 3 SD within the individual subject and 3 SD at the specific site (Haffajee et al., 1983a).

Running medians: this can detect abrupt shifts in attachment level, but an arbitrary standard for change needs to be selected (Haffajee et al., 1983a).

Regression analysis and tolerance methods are the most commonly used in clinical trials. The tolerance method requires measurements as few as 2 points in time and is most suitable in detecting abrupt differences in a short period of time (Haffajee et al.,1983a). The regression analysis method is most suitable for assessing linear changes in attachment level over a long period of time. Haffajee et al.(1983a) evaluated the above statistical methods in detecting periodontal disease activity in individual sites. They found that regression analysis was particularly sensitive to gradual changes whereas the tolerance method was well suited to detecting changes over a short period of time. However, the last technique has

been criticized, because it uses standard deviations as its basis and not actual differences between replicate measurements (Janssen et al.1987). Regression analysis has shown that the number of sites with periodontal breakdown is twice that determined by the other two methods.

Haffajee et al.(1983b) suggested a criterion of 3 standard deviations of the difference of replicate measurements, which was equal to 2.46 mm (rounded to 3mm) in their study, to reduce false positive detection of attachment level change. This technique (3mm threshold) has been criticised by Fleiss et al.(1991) as it has been applied to various populations not having a similar precision of measurement. Lindhe et al.(1983) used > 2mm as a threshold for detecting attachment loss in their study, because they considered that changes > 3 standard deviations (SD=0.83mm reported by Haffajee et al.1983a) to be unlikely to be due to measurement error.

Badersten et al.(1987) used a linear regression analysis for the identification of sites with gain or loss of probing attachment over 2 years, using significance level of $P<0.05$. A minimum projected (change/year) of equal to or >1.5mm (gain or loss) and a probability of $p<0.05$ were used to classify a site as showing probing attachment gain or loss.

Cohen and Ralls (1988) estimated the false positive rates in the determination of changes in probing depth-related periodontal measurements using computerized simulation. They found that one third of sites detected by tolerance

method (using threshold equal to or < 2.5mm) as showing "bursts" of periodontal attachment change may be false positives attributable to measurement error.

Goodson (1986) used computer simulation, he calculated the expected Type-1 error and Type-II error rate for each method and found them to be sufficiently low, while his results for Type-II error suggests that a substantial fraction of disease active sites was not detected by these methods.

End-point analysis: this is used to compensate for the inability of linear regression to detect rapid modulations at the beginning and the end of observation periods. However, this test is based on fewer probings and may include more sites affected by probing error (Badersten et al.1987). In this method the deeper of the pair of initial measurements is compared against the shallower of the final pair of measurements and if the change exceeds a specified threshold, attachment level change is declared.

Evidence for rapid changes in periodontal attachment level and pocket depth is based almost exclusively on differences between sequential periodontal probing measurements. Real attachment losses are postulated to have occurred if changes at or beyond a specified magnitude are present at frequencies substantially above those expected by chance.

Other methods:

Aeppli et al.(1985) used the estimating method of the false positive rate and concluded that observing an increase of probing depth greater than 1mm serves as a diagnostic test

with high sensitivity and specificity.

The criteria adopted by Lindhe et al.(1989b) for considering that a site has lost a significant additional amount of attachment was an increase in probeable attachment level of >2mm. The choice of this threshold was based on estimates of Type 1 error rate and the false positive rate for attachment losses for single measurements presented by Glavind and Loe (1976) and replicate measurements by Gunsollely and Best (1988).

Zappa et al.(1990) investigated the error for probing measurements when assessed using all the sets of double measurements. Clinical disease progression in their study was defined as probing attachment loss reaching or exceeding a fixed threshold of 2 mm.

Gunsolley and Best (1988) estimated the error rates associated with pocket depth and attachment level measurements by using a simulation method based on resampling of replicated attachment level measurements. They concluded that a large proportion of perceived changes in attachment level or pocket depth can be attributed to the false positive rate (the relative proportion of sites with measured change in attachment level that occur in the absence of real change in attachment level). They also concluded that replicating measurements of attachment level reduce the rate of false positives, and found that with a difference in the mean of replicated measurements greater than or equal to 2mm, only 16% of changes were attributable to error.

Best et al.(1990) investigated the reliability of attachment loss measurement in a longitudinal trial, and found that attachment level measurements were in agreement within 2mm on more than 95% of sites and detection of attachment level change with a standard periodontal probe in a longitudinal study can be reliable when 2 examiners each detect a change of 2 mm or more at a subsequent examination. They defined a reliable criterion for change in attachment level which has less than 5% chance of obtaining one false positive change in attachment level for a mouth with 120 sites. Their results point out the lack of sensitivity of standard periodontal probe measurements in the detection of changes in periodontal attachment loss, and 2-3 mm of change appears to be the limit of sensitivity due to the high error inherited in the measuring method. Lindhe et al.(1986) used a safety threshold of more than 2mm of probing attachment level difference to identify sites with attachment loss. The reproducibility studies of Badersten et al.(1984d) suggest a probing attachment loss of more than 2mm occurs on the average in only 1-5% of repeated measurements. On such a basis therefore Lindhe et al.(1986) concluded that this threshold should provide sufficient margin of safety. They used the frequency distributions of the standard deviations of the repetitive measurements at sites which showed no significant attachment level change by regression analysis (change less than 0.5mm at $P<0.05$). They selected a cut off point of >2mm as the safety threshold, based on the chance that

false positive results would be less than 5%. The safety requirement was 2 X SD of those stable sites, and which was within less than 5% of the false positive.

1.5.1.4 Radiographs

In periodontology radiographs are used primarily to estimate the severity and pattern of bone loss, to detect pathological lesions and to study the effects of occlusion (Lang and Hill, 1977). Radiographs give information on hard tissue changes but they are not able to provide information on current disease status or predict future disease activity. They are of no value in the diagnosis of gingivitis and very early periodontitis.

1.5.1.5 The ability of the clinical parameters to predict periodontal disease activity

The ability of clinical parameters (PI, GI, BOP, PD) to predict periodontal disease activity as measured by the loss of attachment level has been investigated in longitudinal studies. However, investigations have failed to report any significant relation between a subsequent significant loss of probing attachment level used as an indication of disease activity, and these clinical parameters (Listgarten and Levin, 1981; Haffajee et al., 1983b; Badersten et al., 1985a, 1987, Fine and Mandel, 1986).

Haffajee et al.(1983b) assessed the diagnostic relationship between certain clinical parameters (BOP, suppuration and PI) measured before each 2-months time interval and future disease activity in untreated periodontal disease patients over a 2-year period, and found that none of the parameters used either individually or in combination was useful in predicting disease activity.

Badersten et al.(1985c,1987) and Vanooteghem et al.(1987) reported the association of certain clinical parameters with the incidence of breakdown sites monitored at three month intervals during therapy and a follow-up observation period of 24 months. They (Badersten et al., 1985c) found that sites showing probing attachment loss were more frequent among sites previously showing high scores for plaque, bleeding on probing, and probing depth than in sites with low scores. These clinical parameters were all associated with probing attachment loss, but their predictability value did not exceed 30-40%, and thus they cannot be used to predict periodontal disease activity. Badersten et al.(1987) found that the initial probing depth could be related to subsequent change in probing attachment level. Sites with deep initial probing depth subsequently demonstrated a high incidence of gain and a low incidence of loss of probing attachment. Probing attachment loss of equal to or >1.5mm was more frequent in shallow sites than deep sites. The PI and BOP showed a weak or no association with probing attachment changes following therapy. Probing attachment loss was more frequent at sites that exhibited

bleeding on probing at the majority of the examinations during 2 years.

Badersten et al.(1985c,1987) and Vanooteghem (1987) showed that when the bleeding frequencies increased, the corresponding predictive values also increased at < 26%., and as the plaque frequency increased, the positive predictive value increased up to 25% (Badersten et al.1985c ,1987).

Lang et al.(1986) in his retrospective study over 4 years with examinations at 3-5 months intervals, found that as the frequency of BOP increased, the positive predictive value of breakdown increased (15% to 30%).

Lindhe et al.(1989) found that the relationship between PI and changes with respect to probing depth and attachment level were weak during a one year observation with examinations at 2 months intervals.

Goodson (1986) found that the clinical measurements (gingival redness,BOP ,suppuration, PI) and DFM bacterial counts exhibited no association with episodic attachment loss. Only the loss of alveolar bone and the presence of specific bacterial species have been associated with episodic attachment loss.

Haffajee et al.(1988) found that the severity of gingival inflammation (GI, BOP, PI) related poorly to mean attachment loss.

Claffey et al.(1990) investigated the diagnostic predictability of scores for plaque, bleeding, suppuration and probing depth for probing attachment loss over 3.5

years following initial periodontal therapy. They found that accumulated PI scores gave low predictability, while accumulated BOP scores gave modest predictive values for attachment loss. After a few years of maintenance, an increase in probing depth, particularly if combined with a high frequency of bleeding on probing, showed the highest predictive value for probing attachment loss of the sites examined.

Okamoto et al.(1988); Yoneyama et al.(1988); and Lindhe et al.(1989a),all found a non-significant relationship between changes in probing depth and probing attachment level.

Badersten et al.(1990) found a modest predictive value for PI and BOP in predicting probing attachment loss. Increased probing depth on the other hand showed high diagnostic predictability, at least during the final years of a five years observation period. Probing attachment loss was most frequently found in sites with initial pocket depth (PD) equal to or > 3.5mm and infrequently found in sites with initial PD equal to or more than 7mm. However, Claffey et al.(1990) found attachment loss was more frequent for sites with PD initially equal to or > 7mm than in shallower sites. In both studies PI and BOP frequencies had modest predictive value for loss of attachment level, and also in both studies residual probing depths had a modest predictive value at 12 months but increased predictive value at later yearly intervals. Increase in probing depth compared to baseline seemed to be the criterion with best predictive value, particularly if combined with bleeding

frequencies equal to or > 75%. So it appears that attachment loss is often associated with increased probing depth.

Kaldahl et al.(1990) evaluated the relationship between the presence of both gingival bleeding and supragingival plaque, and probing attachment loss at sites demonstrating > or equal to 2mm probing attachment loss during a 2-year period of maintenance therapy. They found that gingival bleeding and PI were not prognostic factors of attachment loss.

Badersten et al.(1990) studied the diagnostic value of clinical scoring of supragingival plaque, bleeding, suppuration and probing depth to predict probing attachment loss over 5 years of observation following nonsurgical periodontal therapy. The results showed that all the clinical parameters were associated with attachment loss. The diagnostic predictability of either accumulated plaque scores or accumulated bleeding scores reached a maximum of about 30%. Residual probing depth equal to or > 7 mm reached a predictability of about 50%. An increase in probing depth equal to or > 1mm reached about 80% predictability after 5 years, and thus the increase in probing depth was found to be the most valuable method in predicting probing attachment loss.

Jenkins et al.(1988) investigated the ability of certain clinical parameters (pocket depth, PI and gingival redness) and microbiological criteria to predict periodontal breakdown during a one year period (with examinations at 2-

month intervals) in 11 subjects with untreated advanced periodontitis. They found that neither the PI scores, the presence of gingival redness nor the pocket depth measurements could be used in a predictive capacity for attachment loss on either a site or patient basis.

Haffajee et al.(1991) investigated the association of baseline clinical parameters (PD, baseline attachment level, PI, GI, BOP) of periodontal disease with further attachment loss in untreated periodontal disease patients over the following year. They found that these clinical parameters were strongly associated with risk of new attachment loss (at 2 and 3mm thresholds). Their study demonstrated the strength of relation between prior attachment loss and additional attachment loss, and suggested that other clinical variables are less predictive for additional attachment loss in a subject. Their results and those of others (Albander et al., 1986; Axelsson and Lindhe 1978; Haffajee et al., 1988) have indicated that subjects with large numbers of sites of attachment loss or alveolar bone loss are more likely to exhibit additional attachment loss in the same sites or at new sites. So patients already showing more periodontal destruction are at higher risk for additional disease than those with little or no evidence of destruction.

Concluding remarks:

The studies described above have assessed the ability of clinical parameters to predict attachment loss in patients monitored for 2 years or less, and this may have been too

short period of time to properly study the diagnostic value of clinical signs of disease. In general, as time progressed these parameters provided improved diagnostic predictability. Therefore, longer monitoring periods may need to be used if clinical signs of disease are to reach meaningful diagnostic values.

It can be concluded that the clinical parameters (PI, GI, BOP, PD) used in the assessment of periodontal disease cannot predict active periodontal destruction.

It also could be concluded that an improvement in the reproducibility of attachment level measurements and the detection of attachment level change as well as using frequent attachment level measurements, will result in reducing the false positive number of sites with attachment loss. On the other hand, increasing the number of periodontal sites and the length of studies will give a better chance for detecting disease activity at more periodontal sites. This in turn, with no doubt, will improve the correlation between the disease activity, measured by attachment level, and other clinical parameters. So this may have an influence on the predictive value of the clinical parameters in the assessment of periodontal disease activity.

1.5.2 Biochemical Methods and Gingival Crevicular Fluid (GCF)

The examination of the host response in the periodontium by analysis of GCF is an important approach to periodontal diagnosis and the detection of destructive periodontal disease.

Analysis of GCF constituents might provide an early, biochemical indication of tissue changes that may ultimately manifest clinically as destructive periodontal disease. The analysis of GCF for biochemical markers of disease is one of a battery of adjunctive diagnostic tests used in identifying patients and/or sites at risk for disease progression (Lamster et al., 1989; Fine and Mandel, 1986; Offenbacher et al., 1986,Lamster et al., 1988a and 1988b).

Reliable biochemical methods of periodontal diagnosis must show high sensitivity and specificity, and have a high predictive value for the detection of future attachment loss. These methods have two important aims; to identify those patients at risk for breakdown, and to identify the affected sites or regions in the susceptible individual. The analysis of host response elements in GCF may provide a noninvasive means of monitoring events occurring in periodontal tissues.

1.5.2.1 Gingival crevicular fluid; Definition, Production, Sampling methods and volume measurement

Definition:

The gingival crevicular fluid (GCF) in its most elementary form could simply represent interstitial fluid, changing in the presence of inflammation to inflammatory exudate (Alfano, 1974; Pashley, 1976; Hattingh and Ho, 1980). It is thought that interstitial fluid, even in the absence of inflammation, can pass into the gingival sulcus because of the nature of the dentogingival junction (Cimasoni, 1983). Thus GCF can be considered as an interstitial transudate present within or at the orifice of the gingival crevice. It contains various components derived mainly from four sources, these being (1) Interstitial fluid; (2) products of host cells; (3) plasma-derived molecules; (4) products derived from the subgingival plaque (Cimasoni, 1983). Each of these components contains a multitude of factors each of which could be considered a possible candidate for prediction of disease activity.

Production of GCF:

GCF production has been shown to be closely related to an increase in vascular permeability of the gingival crevicular plexuses underlying the sulcular and junctional epithelium, during the early stage of inflammation (Brill, 1959; Brill and Krasse, 1958; Brill and Bjorn, 1959; and Egelberg, 1966).

Production of GCF in clinically healthy gingivae has been

suggested to be controlled by different mechanisms which have been postulated in two models:

1.Alfano (1974) model: this assumes that a very early or preinflammatory flow of gingival fluid may be osmotically mediated. Within the clinically healthy gingiva, the early deposition of a small amount of subgingival plaque creates limited quantities of macromolecules which may be absorbed either on the surface of desquamating epithelial cells or through phagocytosis. Further accumulation of molecules occurs and they diffuse intercellularly between the epithelial cells to the basement membrane which acts as a major limiting barrier. Accumulation of the macromolecules at the basement membrane create an osmotic gradient and this results in the generation of gingival fluid flow. This osmotically modulated gingival fluid is a transudate and not an inflammatory exudate, but at various times it may progress to a an exudate.

2.Pashley mathematical model(1976): this is based on certain factors which govern fluid distribution across capillary walls. The model implies that GCF production is modulated both by the passage of fluid from capillaries into the tissues (capillary filtration) and by its subsequent removal via lymphatics. When the production of fluid from capillaries is greater than the lymphatic uptake, fluid accumulates as oedema or is removed as GCF. The net flow of GCF depends not only upon the difference between capillary filtration and lymphatic uptake, but also on the filtration coefficients of the junctional and

sulcular epithelia. It is suggested that the net GCF production,in both health and disease, can be accounted for by relative differences in the passage of fluid from capillaries into the tissues, the uptake by lymphatics and the filtration through junctional epithelium into the gingival crevice. These differences are governed by differences in the filtration coefficients of the membranes delineating the different compartments as well as by the differences in hydrostatic and osmotic pressure between capllaries, lymphatics, tissue fluid and sulcular areas. Both the Alfano and Pashley models of GCF production can be postulated as follows: In the absence of inflammation, macromolecules originating from accumulated plaque diffuse intercellularly and reach the basement membrane where they create an osmotic gradient and attract interstitial fluid toward the sulcus. When more macromolecules reach the basement membrane, an inflammatory reaction occurs. The osmotic gradient is then greater but the fluid now permeating the weakened basement membrane and the junctional epithelium is a typical inflammatory exudate. Many experiments on GCF production have been conducted. Brill (1959) used histamine injection in dogs to increase vascular permeability,and collected GCF by paper strips from clinically healthy gingival sites.

Egelberg (1966) applied histamine topically to healthy gingivae ,found increased permeability and large amounts of GCF were collected. These experiments support the concept that GCF is derived from plasma.

Bickel, Cimasoni and Anderson (1985) did not report any fluid recovery from healthy gingivae but they found a low concentration of albumin in GCF during pre-inflammatory plaque accumulation. Based on these experiments, it seems that GCF is produced through leakage of plasma fluids and proteins due to increased permeability of the vascular plexus. The fluid passes through the connective tissue and enters the gingival sulcus through the intercellular spaces of the pocket epithelium.

Concentration of proteins in gingival fluid:

Controversial results have been obtained on the protein concentration in GCF.

Brill and Bronnestam (1960) found that the total protein content of GCF in unstimulated pockets was as low as 1:10 of that of serum, while in stimulated pockets it was only slightly higher. Sueda et al.(1966) showed that GCF contains proteins similar to those found in serum. Bang and Cimasoni (1971) showed that periodontal inflammation is accompanied by varying amounts of GCF according to the severity of the inflammation. Their studies confirm that GCF is an inflammatory exudate carrying high molecular weight components such as proteins. Schenkine and Genco (1977) concluded that the GCF from severely inflamed periodontal tissue appears to represent a mild dilution of serum and that less severe periodontal damage produces a less dilute gingival fluid, while in another study they concluded that the amount of protein increases with the

severity of the inflammation.

Generally the protein concentration of GCF sampled from sites with varying degrees of gingivitis and periodontitis is equivalent to that of serum (Bang and Cimasoni 1971; Biswas et al., 1977; Binder et al., 1987).

A number of studies confirm that GCF in cases of gingivitis contains a similar concentration of total proteins as present in serum (Hattingh and Ho, 1980; Bang and Cimasoni, 1971) and indicate also that the concentration of proteins in GCF collected in the absence of clinical inflammation is lower and similar to that of extracellular fluids. In clinically healthy gingiva very little fluid can be collected and GCF appears to contain a low concentration of proteins. This could represent interstitial fluid generated locally by an osmotic gradient and result from an increased permeability of the gingival venules. This may then progress to a classical inflammatory exudate,containing higher amounts of total protein.

The protein concentration in GCF has been found to range from 22-130 microgram/microliter (ug/uL)(Biswas et al. (1977).

Hattingh and Ho (1980) reported that the protein concentration of GCF collected in the absence of clinically visible signs of inflammation is 93 ug/uL, while its concentration in inflamed gingiva is 69 ug/uL which supports the results of Bang and Cimasoni (1971) who reported a level of 68.3 ug/uL.

Novaes et al.(1980) demonstrated that GCF flow increased

directly with the clinical inflammatory response. They found the concentration of 64.1 ug/uL in their study. They also found a higher total protein content in GCF from severely inflamed pockets but lower protein concentration compared to healthy gingiva, and this was due to a significantly higher rate of GCF flow in clinically severe periodontal disease. Their observations are consistent with those of Schenkine and Genco (1977). The protein probably originates in part from the leakage of plasma proteins and in part from the metabolism of various connective tissue proteins.

Curtis et al.(1988) found that the concentration of protein in GCF at an undisturbed healthy site is of the order of 22 ug/uL. They suggested that the variations in the concentration of GCF protein reported in many studies are due to the duration and method of collection and the evaporative loss from the collection device prior to GCF volume determination which causes a proportionately greater underestimation of the total sample volumes when the starting volume is low. Thus in health when the GCF volume is invariably low, there is a tendency to overestimate the concentration of GCF components.

Methods of collection of GCF:

Various methods have been used for GCF collection,and the choice of a suitable technique depends on the type of analysis required and the amount of GCF required. These methods include:

1.Absorbing paper strips: this is the most commonly used method for measuring the flow of GCF and for the quantitative analysis of GCF components (Golub et al.,1976; Cimasoni and Giannopulo, 1988; Griffiths,Curtis and Wilton, 1988; Curtis et al., 1990; Giannopoulp et al., 1992;Lamster et al.,1988a and 1988b). This method was first used by Brill and Krasse (1958) using both intracrevicular and extracrevicular techniques.

2.Capillary tube or micropipette method: this is based on the use of capillaries which allow the collection of 10-40uL of fluid. Capillaries are used for the collection of a large quantity of fluid (Ishikawa et al., 1972).

3.Gingival washing technique: this involves the ejection of and reaspiration of a known volume of solution into the gingival crevice. The method is used when cellular elements of the fluid are studied (Skapski and Lehner, 1976).

Standardisation and choice of the sampling methods:

Analysis of GCF should follow standardisation of the sampling method, as different methods may influence the amount and constituents of the GCF recovered. Also care must be taken during collection to avoid possible variations due to contamination, irritation and evaporation. When microamounts of fluid are sufficient for collection and analysis and when studying the healthy and diseased crevice, the paper strip appears to be the method of choice even though the insertion of a paper strip into the crevice may cause gingival irritation which may lead to

increased vascular permeability .

To avoid the variability associated with GCF collection by paper strip, certain factors have to be considered. These include sampling time and sampling repetition which have been shown to influence the GCF flow rate and may lead to reduction of GCF volume particularly during repeated sampling (Binder,Goodson and Socransky, 1987; Curtis et al., 1988; Cao and Smith, 1989; Lamaster et al., 1989). Variations in the fluid flow with time during the collection procedure could significantly affect quantitative observations on gingival fluid. Depending on the collection procedure, it has been shown that the amount of fluid production does indeed change in the minutes following the contact of the paper strip with the marginal gingiva. So the time factor has to be taken into in consideration when collecting fluid and analyzing data (Griffiths,Curtis and Wilton, 1988; Curtis et al., 1990). Standardisation of the length of the strip to be inserted in the crevice is necessary as the deeper the insertion then the higher will be the recorded flow rate (Mann,1963). The sampling of GCF by standard paper strip using minimum tissue irritation and the minimum adequate time to collect sufficient fluid for analysis, was the method used in the present study.

Measurement of volume of collected GCF:

Various methods have been used for measuring the volume of collected GCF. These include:

1.The wetted area of the filter paper can be viewed with a microscope fitted with a graticule, directly or after staining in an alcoholic solution of ninhydrin at a concentration of 2% (Egelberg and Asstrom, 1973). The staining, however renders the sample unsuitable for future analysis.

2.Weighing the strip enables the flow of fluid to be measured in milligrams per minute, and this is particularly suitable for measuring large volumes of fluid (Cimasoni and Giannopoulou, 1988).

3.The Periotron: is the most suitable method for measuring the volume of GCF absorbed on a paper strip and was developed by Harco Electronics (Dental Products Division, Winnipeg,Canada). The method is compatible with subsequent chemical analysis (Hattingh and Ho 1980) and keeps evaporation to a minimum. It does not suffer from the disadvantages of the ninhydrin technique. The Periotron may be considered an accurate instrument, provided that a daily check of the reading accuracy is performed and paper strips are placed in the machine in a standard position.

The Periotron reading represents the moistened area of the strip rather than the amount of the fluid absorbed. The reading is affected by many factors including the humidity, temperature and most importantly the position of the strip between the jaws of the Periotron. The instrument functioned by measuring the reduction in capacitance between sensors due to the wetting of a precut filter paper strip. This activity was coupled to a digital readout on a

scale of 0-200. The instrument allowed for rapid volume determination, and the availability of the sample for subsequent biochemical analysis. Recently, Periotron 6000 was introduced, with less variability than either the Peroitron 600 or the ninhydrin staining method (Hinrich et al.1984). The Periotron 6000 has been extensively used for the investigation of GCF components and has shown satisfactory results in the assessment of GCF volume (Cimasoni, 1983). The only difficulty is the assessment of minute amounts of GCF (volumes of less than 0.2uL). The measurement of these small volumes is necessary in certain protocols, and be can be subject to uncontrolled errors (Lamster et al., 1988a).

GCF volume flow rate in periodontal health and disease activity:

Many studies have investigated the correlation between GCF flow and the severity of gingival inflammation.

Brill and Bjorn (1959) concluded that inflamed pocket epithelium yielded greater amounts of GCF than healthy epithelium. Mann (1963) concluded that GCF flow correlated with the severity of inflammation, and was able to find a correlation between GCF flow and pocket depth. Egelberg (1966) demonstrated that small amounts of GCF can be recovered from clinically healthy gingiva. He found a positive correlation between gingival indices and GCF flow. Bjorn et al.(1965) found a positive correlation between GCF flow and the gingival index scores.

From the above studies it can be concluded that the volume of GCF can be used as an accurate estimation of the clinical degree of gingival inflammation.

Many differences in GCF quantity and composition from 'healthy' and diseased gingival sites have been identified (Cimasoni, 1983; Fine and Mandel, 1986).

Smith et al.(1991) found striking differences in the volume of GCF from different sites.

Determination of the volume of GCF collected on filter paper strips represents an accurate assessment of early inflammation and vascular permeability in a particular patient. However, the interpatient variability is high, and precludes the use of predetermined volume thresholds to differentiate disease categories. Moreover,there are no data to support the concept that GCF volume can be used to predict future attachment loss.

1.5.2.2 Constituents of GCF and disease activity:

A limited number of markers in GCF associated with progressive attachment loss such as PGE_2 and B-glucuronidase (neutrophil granule enzyme) have been identified (Fine and Mandle,1986; Offenbacher et al.,1986). The evaluation of PGE_2 as a screening test for progressive attachment loss indicated a high sensitivity, high specificity, and high predictive value. Elevated levels prostaglandin E_2 in GCF has been found in patients with periodontitis compared to those with gingivitis (Offenbacher and Goodson, 1984), and deteriorating periodontal sites showed a five-fold

elevation PGE_2 levels compared to stable sites (Offenbacher et al., 1986).

B-glucuronidase was found to be an indicator and predicator of localized and generalized clinical attachment loss with high specificity and sensitivity (Fine and Mandel, 1986; Offenbacher et al., 1986). In a study of patients with periodontitis,B-glucuronidase levels in GCF were predictive for patients and sites about to undergo significant probing attachment loss, with a sensitivity and specificity of 89% (Lamster et al., 1988a,b).

Host cells and their products, inflammatory cells (PMNs) and their enzymes and other substances related to inflammation and tissue destruction have been found in GCF and associated with periodontal diseases (Cimasoni, 1983: Fine and Mandle, 1986). Among those host factors that have been identified in GCF are elastase, various cathepsins, lactoferrin, and myeloperoxidase. Indicators of cell death in GCF include aspartate aminotransferase and lactate dehydrogenase. GCF collagenase activity was found to be increased during active periodontitis in beagle dogs, and was strongly correlated with attachment loss (Kryshtalskyj et al., 1986). It was also found that collagenase activity from periodontal pockets was positively related to GCF flow and pocket depth (Golub et al., 1976).

Factors for complement pathways have also been identified in GCF. The numbers of polymorphonuclear leukocytes in GCF also increases during the development of experimental gingivitis in man (Thurre et al., 1984).

1.5.3 Microbiological methods

Microorganisms are the primary aetiologic agents for the periodontal diseases (Socransky, 1977; Socransky, 1970) and there are hundreds of different species of bacteria present in the oral cavity. Studies, using improved microbiological methods, have confirmed that varying degrees of specificity exist in the bacterial flora associated with health, gingivitis and various forms of periodontitis (Listgarten, 1988; Slots, 1986a).

Microbiolgoical assessments for specific microorganisms in individual periodontitis patients may be applied to determine the causative agent; to assess disease activity; to monitor the effect of treatment; and to decide on recall intervals. It may also be helpful to identify those persons at risk for either the initial episode or for recurrent periodontal disease (Genco et al., 1986). Methods of microbiological assessment include culturing, immunologic assays,DNA probes,enzyme tests,and microscopic methods (Slots, 1986b; Greensten, 1988).

1.5.3.1 Microscopic Methods:

Microscopic monitoring of the subgingival flora by either phase-contrast microscopy (PCM) or differential dark-field microscopy (DDFM) may help when used with other clinical parameters in the diagnosis and management of chronic periodontal disease and may enable the setting of appropriate recall intervals for maintenance therapy. Both

techniques are simple and allow the rapid classification of bacterial samples on a percentage basis according to the shape, size and motility of the bacteria.

Since bacteria can be visualized regardless of their ability to grow in culture, darkfield or phase-contrast microscopy provides a good means of determining both total bacteria counts and counts of bacteria which have characteristic morphotypes. Darkfield/phase-contrast microscopy does not differentiate between microbial species, nor between pathogens and non-pathogens for bacteria with similar morphologies (Greensten and Polson, 1985). For example bacteria such as actinobacillus actinomyecetemcomitans (Aa) and P.gingivalis, are neither motile nor spirochaetal and cannot be specifically identified using microscopic monitoring. Another disadvantage of DDFM and PCM is that the samples must be assessed immediately and cannot be stored for reassessment. Problems due to sampling, specimen dispersion, morphotype classification, and slide preparation have been found to be associated with microscopic studies (Omar and Newman,1986). It has been suggested that microscopic monitoring for elevated levels of spirochaetes and/or motile rods may identify sites of active periodontal breakdown but recent finding do not support this hypothesis (Dunham et al., 1985). However subjects with treated periodontitis and showing elevated levels of spirochaetes and/or motile rods, have more frequent localized recurrences of periodontitis than patients showing low proportions of these morphotypes

(Listgarten and Hellden, 1978).

In subjects who discontinued professional maintenance, the sensitivity and specificity of the microscopic test to predict future breakdown in subjects was close to 80%. However the test was not able to predict which particular sites would be affected.

Microscopic monitoring of spirochaetes and motile forms has been found to be an unreliable predictor of gingivitis (Listgarten, 1985), and inflammatory exacerbations in pockets (Listgarten and Hellden, 1978), and is inconsistent in predicting increasing pocket depths (Listgarten et al., 1984). Microscopic monitoring is not suitable to monitor disease activity (Dunham et al., 1985; Listgarten et al., 1986).

It can be concluded that DDFM and PCM can identify bacterial morphotypes but not specific bacteria, and may be valuable diagnostic aids in the prevention of disease recurrence, but not as predictors of the disease activity.

Microscopic studies of periodontal microbiota have demonstrated the existence of distinctive microbial populations in association with various stages of periodontal disease and periodontal health. This was first clearly established by Listgarten (1976) who on the basis of electron microscopic studies reported many more spirochaetes, Gram negative, and flagellated species in periodontally diseased than in healthy sites. Most of the microbial variance originated between subjects, rather than between sampled sites within each subject (Evian et

al.(1982).

1.5.3.2 Microscopic studies of subgingival microflora in periodontal disease and their relation to clinical parameters

A number of studies using DDFM and PCM have reported distinct differences between the composition of the microbiota at periodontally healthy sites and sites with varying types of periodontal disease (Listgarten, 1986a,b; Loe et al., 1965; Theilade et al., 1966; Listgarten et al., 1975,1976; Listgarten and Hellden, 1978; Lindhe et al., 1980; Keyes and Rams, 1983). Healthy sites were associated with fewer bacteria, predominantly consisting of coccoid forms and non-motile bacteria with greatly reduced numbers of motile rods and spirochaetes (Listgarten and Hellden, 1978). Diseased sites were associated with increased numbers of bacteria, with motile forms especially spirochaetes, and with decreased numbers of coccoid forms(Listgarten and Hellden 1978).

Several workers have shown a clear association between changes in the proportions of the different morphologic types and the disease activity, particularly the correlation between increases in the proportions of spirochaetes and other motile rods and increased disease severity (Lindhe et al., 1980; Listgarten and Levin, 1981). Spirochaetes and motile forms have been frequently found at diseased sites (Listgarten and Hellden 1978; Armitage et

al., 1982; Listgarten and Schifter 1982; Savitt and Socransky 1984; Greenwell and Bisada 1984; Lindhe et al., 1980), but their proportions were best correlated to pocket depths (Mousques et al., 1980; Armitage et al., 1982; Evian et al., 1982; Listgarten and Levin, 1981; and Savitt and Socransky, 1984). Other have also demonstrated a correlation between these forms (spirochaetes and motile form) and pocket depth (Keyes et al., 1978; Listgareten and Hellden 1978; Lindhe et al., 1980; Listgarten and Levin, 1981; Armitage et al., 1982; Savitt and Socransky 1984; Green and Bissada, 1984). Evian et al.(1982) found a lack of correlation between clinical parameters (PI, GI, PD) and the proportions of spirochaetes and motile rods at individual sites.

Listgarten and Hellden (1978) reported significant differences in the microbial flora between clinically normal and diseased sites within the same patient using DDFM. They found that the proportions of cocci were higher at normal sites (74.3%) than at disease sites (22.3%). Motile rods were more numerous at diseased sites (12.7%) than at normal sites (0.3%) and spirochaetes comprised 37.8% of the bacteria at diseased sites compared with 1.8% at normal sites (relatively healthy sites with only mild evidence of clinical inflammation). The ratio of motile to non-motile bacteria in normal sites was found to be 1:49,whereas in diseased sites the ratio was in the vicinity of 1:1. The findings indicated that diseased sites were associated, with an increased number of motile rods

and spirochaetes with a decrease in the number of coccoid forms. These results have been supported by Lindhe et al.(1980) who found an increase in motile forms and a decrease in coccoid forms with increasing severity of periodontal destruction.

Keyes and Rams (1983) monitored the composition of subgingival flora by PCM and found that spirochaetes and motile rods were highly associated with destructive periodontal disease.

Mousques et al.(1980) using DDFM reported a moderately strong correlation between the percentages of spirochaetes and GI, PI, and PD.

Listgarten and Levian (1981) found a positive correlation between the percentage of motile rods and GI and PI, and between spirochaetes and PI and PD, with a negative correlation between the percentage of cocci and PI when sampled surfaces only were considered for analysis. They concluded that there was a positive correlation between the proportion of both subgingival spirochaetes and motile rods with susceptibility to periodontal deterioration.

Evian et al.(1982) found no significant correlation between clinical parameters (PI, GI, PD) and the proportions of spirochaetes and motile rods at individual sites. They suggested that bleeding on probing is positively correlated with the proportion of subgingival spirochaetes.

Savitt and Socransky (1984) using DDFM found that spirochaetes and other motile organisms were more frequently present and in higher proportions in gingivitis

and chronic periodontitis than in healthy subjects,and the proportions of these bacteria were highly correlated with pocket depth. Motile organisms were also found to be positively correlated with PI and GI,in contrast to cocci which showed a strong negative correlation with pocket depth, PI and GI. Their results support the findings of Armitage et al.(1982) that the percentages of spirochaetes and motile rods may be related to the pocket depth.

Greenwell and Bissada (1984) reported the presence of variations in the subgingival microflora from healthy and intervention sites using DDFM and probing depth criteria. Means from all sites indicated that deeper pockets were associated with increased proportions of spirochaetes and motile forms. They supported the concept of site-specific variations in the subgingival microflora of patients susceptible to periodontitis.

Wilson et al.(1985) using DDFM found a significant relationships between the composition of the plaque and probing depth and also with bleeding on probing at the associated periodontal site. A close correlation between clinical symptom "bleeding on probing" and the percentage of motile microorganisms in the subgingival plaque was evident. They confirmed that the proportion of motile rods is not related to actual severity of disease as measured by probing depth.

Bleeding upon probing has been shown to be a reliable parameter for predicting a subgingival microflora with an increased proportion of motile bacteria (Muller et al.,

1986). Similar observations were made by Armitage et al.(1982) who showed that BOP correlated positively with a subgingival microflora with increased proportions of motile micro-organisms particularly the spirochaetes.

Wolff et al.(1985) using DDFM and PCM in a cross sectional study supported the conclusion of other investigators (Listgarten and Levin 1981, Armitage et al.1982) that higher proportions of spirochaetes occur at deeper PD.

MacPhee and Muir (1986) found that deeper pockets tend to contain higher percentages of spirochaetes and motile organisms.

Abbas et al.(1986b) using PCM found that subgingival samples from experimental gingivitis contained a high proportion of spirochaetes. No differences in spirochaetes, motile rods, and other forms could be demonstrated between bleeding and non-bleeding pockets, and this is in agreement with Evian et al.(1982).

1.5.3.3 Microscopic assessment in prediction of periodontal disease activity (Attachment Loss)

Many studies have been conducted to evaluate the role of subgingival morphotypes particularly spirochaetes and motiles in predicting the disease activity measured by attachment level loss.

Studies on periodontal disease activity (PDA) and an association with bacteria have found that microbial determination has contributed limited information to

facilitate PDA prognostication (Greenstein and Polson, 1985; Greenstein, 1988). Retrospective studies showed that spirochaetes cannot forecast PDA (Listgarten et al., 1986; Macfarlane et al., 1988).

Listgarten et al.(1978) suggested that spirochaete counts may not be good predictors of attachment loss. Positive correlations have been reported between the proportions of spirochaetes and motile organisms in subgingival plaque and susceptibility to periodontal deterioration.

Listgarten and Levin (1981) found that the presence of elevated proportions of spirochaetes may predict further periodontal breakdown. This was confirmed in part by Slots et al.(1985) who found a significantly positive correlation between spirochaetes and further loss of periodontal attachment (equal or > 2mm) at 3-6 months and 12 months. Claffey et al.(1985) showed that spirochaetes related better to probing depth than to changes in probing attachment level 12 months after therapy.

Baab and Opsving (1986) using PCM did not find a significant differences in the percentages of cocci, motile rods, or spirochaetes at bleeding and nonbleeding sites. In contrast significant correlations were found between the percentage of spirochaetes and PD, attachment level, and gingival inflammation.

Necomb and Nexon (1989) found a significant negative correlation between probing depth or attachment loss and the proportion of cocci in the plaque, no significant association being observed between clinical signs such as

BOP and the microbiological assays. Their studies are in agreement with those of Baab and Opsvig (1986) who found no differences in the proportions of spirochaetes, motile rods, and cocci between bleeding and non-bleeding sites of similar probing depth.

MacFarlen et al.(1988) investigated the possible correlations between attachment level change and spirochaetes at baseline visit, at 2 months and after one year in patients with untreated periodontitis. They concluded that the quantification of neither spirochaetes nor black-pigmented bacteroides species can be used reliably to identify or predict disease-active sites.

Bacterial products, host cells and their derivatives were found unable to forecast PDA (Fine and Mandel, 1986).

1.5.4 Immunological methods:

Studies of immunological factors in periodontal diseases have led to advances in understanding the pathogenesis of these diseases. Immunological techniques may also help in the identification of bacteria important in the aetiology of periodontal disease. The immunologic methods of diagnosis include assessment of immunoglobulins both in serum and in GCF, assessment of peripheral blood and gingival lymphocytes, and evaluation of phagocytes, including monocytes and neutrophils. These studies have led to recognition that the neutrophil phagocyte is a key protective cell against periodontal infecting bacteria.

1.Antibodies: these include both serum and GCF immunoglobulin levels to putative periodontal pathogens or marker organisms. Elevated serum immunoglobulin titres to A.actinomycetemcomitans have been reported for localized juvenile periodontitis (Genco et al., 1985), and to B. gingivalis for adult and rapidly progressive periodontitis (Slots, 1979; Chung et al., 1983). Reasonable correlations have been reported between elevated serum immunoglobulin titres and specific organisms associated with active lesions (Ebersole et al., 1987).

Studies of GCF antibodies have demonstrated that immunoglobulin levels in GCF are generally lower than in serum, but may be higher in about 27% of diseased sites when antibody response to particular periodontopathic organisms are assessed (Taubman et al.1982). This supports

the concept of either local antibody production or the existence of a concentration mechanism at diseased sites. The antibody studies have helped in understanding the infectious nature of periodontal disease. Antibacterial immunoglobulins, especially those in GCF may be important in controlling subgingival levels of periodontal bacteria, localizing them to the periodontium. Specific immunoglobulins are thought to exert their protective effects by opsonising periodontal bacteria, preparing them for phagocytosis and killing by neutrophils.

2.Neutrophils:

These include the defects in polymorphnuclear leukocytes and/or monocyte function (chemotaxis, phagocytosis) as have been found to be associated with chronic periodontitis (Altman et al., 1985). In vivo crevicular leukocyte tests involving a chemotactic challenge are apparently useful for determining neutrophil function (Golub et al., 1981).

3.Lymphocytes:

Studies of lymphocyte function includes their involvement in the production and activity of biologic response mediators. Autologous mixed lymphocyte reaction (AMLR) was found to be associated with early onset periodontal disease (Ranney et al., 1981).

1.6 Acute phase response

1.6.1 Introduction

The acute phase response is the response of the body to injury or trauma. It consists of an orderly and orchestrated series of reactions that normally result in the arrest of the process of injury, in protection of the organism against further injury, and the initiation of repair and restorative processes aimed at returning the organism to a homeostatic balance.

Immediate local responses to injury include the release of potent components from activated and damaged cells such as lysosomal enzymes and products of the arachidonic acid pathway.

Systemic responses are slower and include activation of phagocytic cells and the release of cytokines and hormones from various cells. Also occurring are changes in the metabolic activity of the liver which result in the increasing synthesis and secretion by hepatocytes of a number of plasma proteins termed "the acute phase proteins"or "acute phase reactants".

Many of the acute phase proteins are present normally in plasma and their levels only slightly increase during the acute phase response. Examples of such proteins include proteinase inhibitors particularly alpha-1-antitrypsin, fibrinogen, complement (C1-C6 ,C9), alpha-1-macroglobulin, alpha-2- macroglobulin. Other acute phase protein such as

serum amyloid, and particularly C-reactive protein (CRP) increase dramatically (100-1000 fold) within 24 to 48 hours after the injury. The levels of other acute phase proteins may even decrease, examples being albumin, lipoprotein and transferrin, and these are termed "negative acute phase proteins".

The term "Acute phase response" was first introduced by Abernethy and his colleagues in 1941, to refer to the changes in serum obtained from patients with infectious diseases (Abernethy and Avery 1941).

The acute phase response is the response by an organism to disturbance of its homeostasis. This may be due to diverse stimuli such as tissue damage, trauma (physical and chemical), infections (bacterial,viral, fungal, parasitic), ischemic necrosis, neoplasms, immunologic disorders and other insults. These stimuli are considered as having a common denominator for production of cellular/tissue injury or death.

The acute phase response is thought to be beneficial to the injured organism with the aim of restoring the disturbed physiological homeostasis. The increased levels of acute phase proteins are an important component of the systemic response to local or general injury, which also includes cellular, microbiological, biomedical, endocrine, and other metabolic changes such as leukocytosis and fever.

The acute phase response is well preserved throughout phylogeny and represents an important mechanism in mammalian systems. The acute phase response often shows a

striking species specificity, CRP for instance being an acute phase protein in man but not in rats or mice, while alpha 2-macroglobulin is an acute phase protein in rats but not in man (Fey, 1987).

The non-specific nature of the acute phase response is an indication of its general activation and participation in both innate and acquired immune reactions.

1.6.2 Induction and regulation of acute phase response and protein synthesis

The acute phase response refers to changes in the concentrations of a large number of plasma proteins which reflect the re-orchestration of a pattern of gene expression for secretory proteins in hepatocytes (Gauldie et al., 1989). It is thought to be co-ordinated by signals released at the site of injury which are able to circulate through the blood stream to remote sites.

The increased levels of acute phase proteins are mainly due to their increased synthesis, although in some cases there may be a contribution from the release of preformed stocks of protein (Koj, 1974). Most acute phases proteins are synthesized by hepatocytes, and their increased production results from an increased number of cells being recruited to active synthesis. The acute phase response is mediated by heterogeneous mechanisms (Kushner et al., 1989).

The regulation of the synthesis of the acute phase response appears to be highly complex and is widely modulated by several specific cytokines. These cytokines are synthesised

and released in response to an inflammatory stimulus by a variety of cells which are present at the inflammatory site, such as macrophages, fibroblasts, endothelial cells and keratinocytes. The cytokines involved are interleukin-1 (IL-1a and IL-1b), interleukin-6 (IL-6)/interferon B2 (INFB2), tumour necrosis factor-a(TNF-a), IFN-gamma, transforming growth factor-B (TGF-B), and leukemic inhibitory factors. These cytokines either alone or in combination have been shown to be capable of influencing plasma protein synthesis in human hepatocytes (Gauild et al., 1987; Ramadori et al., 1985; Perimuter et al., 1986; Mageiaiska-Zero et al., 1988; Baumann et al., 1989; Mackiewicz et al., 1990).

Gauldie et al.(1989) demonstrated that the factors derived from monocytes which stimulate hepatocytes can be accounted for by three polypeptide cytokines; the major hepatocyte-stimulating factor (HSF) activity is encompassed in the molecule known as IFN-B2/BSF2/26KDa protein /HGF/HSF/IL-6 and minor HSF regulation or modulation is delivered by IL-1 and TNF. Each of these polypeptides has specific receptors on the hepatocyte. The acute phase protein genes in the hepatocytes can be separated into subsets that are regulated by IL-6 alone or in combination with other peptide hormones, and this is evidence of the additive or negative effects of the "alarm cytokines" on IL-6 mediated induction of genes expression.

IL-6/BSF2/INFB-2 has been implicated as a potent HSF that drives the increased production of some acute phase

proteins. IL-1B as well as TNF-a has no stimulatory effect on human CRP expression while IL-6 stimulates the full spectrum of acute phase proteins including CRP (Castell et al., 1988). This implies that IL-6 has a key role in the synthesis of human acute phase proteins.

Studies by Andus et al.(1987,1988) showed that recombinant IL-6 (rIL-6/BSF2/IFN-B2) is the major regulator of in vitro hepatic acute phase gene regulation. IL-6 in vivo should cause an acute hepatic phase response similar to that in vitro (Nijstan et al., 1987; Vavoers et al., 1988).

Castell et al.(1989a,b) stated that activated monocytes synthesise and release the cytokines IL-1, IL-6, and TNF-a, with only IL-6 being able to induce the full spectrum of acute phase proteins. They further stated that due to induction and secretion of IL-6 in fibroblasts by TNF-a and IL-1, a further amplication of acute phase proteins induction is possible.

Factors other than cytokines (i.e. co-factors) such as corticosteriods, may be required for optimal induction of several acute phase proteins. As IL-1 and IL-6 stimulate adrenocorticotrophic hormone (ACTH) synthesis (Wolski et al., 1985), glucocorticoids are synthesized which in turn stimulate acute phase proteins synthesis (Gross et al., 1984). Increased glucocorticoid concentrations inhibit the production of IL-1, IL-6, and TNF-a (Lew et al., 1988). Steel et al.(1991) found that acute phase proteins in vitro differ in their responses to IL-1B and IL-6 alone, and in combination. Dexamethasone enhanced the cytokine-driven

induction of a subset of acute phase protein genes. Both cytokines have an additive or synergistic effect on C-RP and serum amyloid A (SAA) when added in combination with dexamethasone.

1.6.3 Interleukin-6 and the acute phase response

Although several cytokines participate in the induction of acute phase proteins synthesis, most of the studies carried out in vitro and in vivo using either hepatoma cell lines or human hepatocyte cultures point to the specific role of IL-6 in the synthesis of the full spectrum of acute phase proteins.

Interferon-B2 (INF-B2) has been shown to be identical with B-cell stimulatory factor-2 (BSF-2). This is a 26kDa protein and the name IL-6 was proposed for this molecule which is identical to hepatocyte-stimulating factor (HSF) (Gauldie et al., 1987.

IL-6 is synthesized by many different cells such as fibroblasts and monocytes/ macrophages. Endothelial cells are probably the major sources of IL-6 during systemic inflammation. IL-6 acts on different target cells in vitro, but in vivo the liver is the major target organ.

Studies on human cell lines show that IL-6 is an inducer of the acute phase response, and stimulation of HepG2 cells line by recombinant IL-6 resulting in an increase in the synthesis of a1-antitrypsin but not CRP or serum amyloid A. The combination of IL-6 and IL-1 however, stimulate production of CRP and serum amyloid A by Hep3B cells. Only in NPLC/PRF/5 cells could an IL-6-dependent stimulation of

CRP and SAA synthesis be detected (Ganapathi et al.,1988a). Studies using human hepatocyte primary culture showed that IL-6 is the major inducer of acute phase protein synthesis, and is capable of inducing CRP and SAA synthesis in a dose and time-dependent manner(Castell et al., 1988). Studies by Moshage et al.(1989), and Castell et al.(1989b) (Castell et al., 1989a) showed that IL-6 stimulated the full spectrum of acute phase proteins seen in inflammatory states in man, while IL-1 and TNF-a had only a moderate effect on the positive acute phase proteins and they failed to induce CRP and SAA. This strongly suggests that IL-6 plays the key role in human acute phase protein synthesis. Hinrich et al.(1990) concluded that IL-6 is a major mediator in the hepatic acute phase response.

Monocytes/macrophages when stimulated by bacteria, lipopolysaccharides or viruses synthesise, secrete IL-1, IL-6, and TNF-a which act via specific hepatic receptors. IL-1 and TNF-a also stimulate fibroblasts, endothelial cells and keratinocytes to synthesize IL-6 and thus amplify the biological effects. IL-6 and TNF-a have been shown to stimulate the release of ACTH from pituitary cells, leading to increased secretion of glucocorticoids by adrenocortical cells. The glucocorticoids on one hand increase the stimulatory effect of monokines on the synthesis of acute phase proteins by hepatocytes, while on the another hand inhibit the synthesis of monokines.

It could be concluded that IL-6 is the most potent inducer of acute phase protein synthesis in human hepatocytes.

1.6.4 Regulation of C-reactive protein synthesis

CRP synthesis is regulated in a similar way to the other acute phase proteins during the acute phase response. Various experimental studies have been carried out in vivo and in vitro using human hepatoma and hepatocyte cell line cultures which strongly point to a key role of IL-6 in the regulation of CRP synthesis.

Induction of CRP synthesis in a human hepatoma cell line culture was reported by Darlington et al.(1986) and Goldman et al.(1987), who showed that IL-1 and IL-6 are able to stimulate CRP synthesis.

It has been found that CRP induction required cytokines other than IL-1 or TNF-a and neither human rIL-1 nor human rTNF-a caused induction of CRP in Hep3B cells (Taylar et al., 1989; Castell et al., 1989b; Ganapathi et al., 1988b). Castell et al.(1988), and Moshage et al.(1988) found that IL-6 enhanced the synthesis of both CRP and SAA in human primary hepatoma cultures, although Morrone et al.(1988) demonstrated that IL-6 had only a minimal effect in enhancing gene expression associated with CRP synthesis.

Studies by Ganapathi et al.(1988a) showed that partially purified IL-6 and rIL 6 increased the synthesis of CRP in the NPLC/PRF/5 cell line in a concentration-dependent manner but not in the Hep3B cell line.

Studies of the effect of IL-1 in combination with IL-6 on the induction of CRP revealed that the addition of either IL-1a or IL-1b did not further increase the response of CRP

in the NPLC/PRF/5 cell line above that observed with IL-6 alone, but that in the Hep3B cell line the combination of IL-1a or IL-1b with IL-6 led to substantial induction of CRP synthesis. Their findings demonstrated that CRP induction in NCLP/PRF/5 cell line is not mediated by autocrine secretion of IL-1, and they demonstrated that IL-6 plays an important role in regulating the synthesis of CRP in human hepatoma cell lines.

Studies of Taylor et al.(1989) showed that the induction of CRP synthesis, by both rIL-1 and rIL-6, is significantly different in hepatoma cell line PLC/PRF/5 than in Hep3B-2 and HepG2 cell lines to the same range of concentrations of each cytokine. They suggested that both IL-6 and IL-1 induce new CRP synthesis and may function *via* distinct mechanisms.

Goldman et al.(1989) showed that *in vitro* human hepatoma cell system, the CRP *de novo* synthesis could be secreted under the induction of certain factor. This factor could be released from endotoxin-stimulated peripheral blood monocytes or from human T-cell lines obtained from T-cell lymphoma patients. This factor has been named hepatocyte stimulating factor type 2 (HSF-2) and is known to be synonymous with IFN-B2,BSF-2, and IL-6. Thus IL-6 plays a major role in the tissue-specific induction of particular acute phase proteins such as CRP.

The study of Moshage et al.(1988) indicated that rIL-1 and rIL-6 both stimulate the secretion by liver of CRP in an additive manner and that IL-1 exerts its action on enhanced

synthesis of CRP at least in part via IL-6.

Castell et al.(1989b) showed that IL-6 stimulates the synthesis of the full spectrum of acute phase proteins including CRP.

It has been concluded from these studies that IL-6 plays the key role in enhanced secretion and regulation of acute phase proteins synthesis, including CRP by human hepatocytes.

Castell et al.(1990) suggested that rIL-6 exerts its regulatory effect on CRP synthesis at the pretranslation level. IL-6 which is synthesized and secreted by human monocytes, fibroblasts and endothelial cells on stimulation by IL-1B and TNF-a, is capable of inducing CRP gene expression and CRP synthesis.

Taylor et al.(1990) showed that transforming growth factor-B (TGF-B) released by platelets early in the inflammatory response modified the production of CRP induction by IL-1B and IL-6, but it inhibits the IL-6 induced-synthesis of CRP at a post-transcriptional level in hepatoma cell lines.

A2-Macroglobulin (A2-M) has been shown to bind IL-6,but the bound IL-6 still retains its biological activity (Matsuda et al.,1989). It binds transforming growth factor-B (TGF-B) (Oconnor-McCourt et al., 1987) which then becomes inactive, regaining its activity only upon release from the complex. Taylor et al.(1991) reported that A2-M can completely neutralize the inhibitory effect exerted by TGF-B on the IL-6-induced CRP production by human hepatoma cell line PLC/PRF/5 without affecting the induction of CRP synthesis

by IL-6. The interaction between A2-M and TGF-B may influence the production of acute phase proteins by liver hepatocytes. A2-M binds non-covalently with TGF-B to form a complex which upon acidification releases TGF-B inhibitory activity as detected by IL-6-induced CRP production.

Certain studies have pointed to the presence of co-factors such as corticosteroids which enhanced the induction of CRP by cytokines in a dose-dependant fashion in Hep3B cell cultures. Caffeine also acts as a co-factor in the induction of CRP by IL-6 and IL-1 in Hep3B cells, and by IL-6 alone in NLPC/PRF/5 cells (Kushner et al., 1989). Studies by Ganathapi et al.(1989) showed that the induction of CRP in the Hep3B cell line required the combination of dexamethasone and insulin while neither cytokine alone induced a significant increase in the synthesis of CRP. It has been shown that IL-6 alone is sufficient for CRP production in the presence of dexamethasone in NPLC/PRF/5 (Ganapathi et al., 1988b).

Further studies by Ganapathi et al.(1991) in Hep3B cells, showed that IL-6, IL-1a and dexamethasone can influence CRP synthesis, while TNF-a alone has no significant effect on CRP. A detectable effect of IL-6 plus TNF-a was not observed on CRP synthesis and an increase in the concentrations of either IL-6 or IL-1a lead to a dose-related increase in CRP synthesis as long as a minimal amount of the other cytokines was present. Dexamethasone alone had no effect on CRP synthesis. These studies are in

agreement with Ganter et al.(1989) studies which showed that TNF-a acting alone or in combination with IL-6 has no effect on CRP expression, and the main modulator of CRP expression is IL-6. Ganter et al. (1989) also showed that IL-6 action is not enhanced by IL-1B which is unable to exert any relevant effect.

1.7 C-reactive protein (CRP)

1.7.1 Historical review

Tillett and Francis (1930) observed that sera from patients with acute febrile illness reacted with a C-polysaccharide (CPS) known as fraction C or C-substance, extracted from the cell wall of Streptococcus pneumonia. Their experiments showed the early appearance of a precipitin for this C-substance and that this precipitation reaction is not limited to pneumococcal infections. The substance responsible for this precipitation was termed C-precipitin and was found to occur in a variety of febrile and non febrile bacterial diseases as well as in non infectious states.

Abernethy and Avery (1941) reported that this reactive substance was a protein in nature and confirmed the results of Tillett and Francis (1930). They showed that this reactive substance is capable of precipitating in the presence of calcium the C-polysaccharide in the sera from patients with a variety of diseases in the acute phase stage and considered it as an abnormal protein present only in the acute phase sera associated with a variety of pathological conditions but not in healthy sera; their reports led to its designation as "C-REACTIVE PROTEIN" (CRP).

Macleod and Avery(1941) described an antibody to this CRP raised in the rabbit called anti-rabbit CRP, and showed

that sera from healthy subjects are free from CRP.

Anderson and McCarty (1951) confirmed the occurrence in the rabbit of an acute phase protein analogous to human CRP which reacts with Cx-polysaccharide in the same manner as the CRP reaction with C-polysaccharide. They designated it as Cx-reactive protein (CxRP).

CRP was first found in normal sera in a trace amounts by Seligman et al.(1959) and this was confirmed by Goldwasser and Rozansky (1961). Nilsson (1968) showed that CRP is present from trace amounts to 4mg/l in sera of healthy subjects.

C-reactive protein is a normal human plasma protein which is present in the sera of healthy subjects, and in the cerebrospinal fluid (CSF), while it has been reported to be present also in oral fluids such saliva and gingival crevicular fluid (Aziz et al., 1990).

It is considered as a protype acute phase reactant as it is the first protein that increases during the early stage of the acute inflammatory response in most forms of injury, infections, inflammation or tissue damage. It increases dramatically and rapidly reaching 100-1000 folds of its normal serum concentrations within 48 hours of an acute stimulus such as serious trauma or severe inflammatory conditions as in the case of significant bacterial infections or active non-infective inflammatory diseases (Kaplan, 1982; Pepys and Baltz, 1983).

Many diverse functional effects of CRP have been reported in vitro and in vivo. The proposed immunomodulatory

functions of CRP are derived from its interactions alone or via its ligands such as C-polysaccharide (CPS), polyanions, polycations, lipids and lipolipids with various elements participating in the inflammatory response. These elements include polymorphonuclear neutrophils(PMN), macrophages/ monocytes, lymphocytes, complement and finally platelets. CRP is now a useful diagnostic aid in many diseases particulary fetal diseases such as cystic fibrosis, rheumatic fever, rheumatoid arthritis and myocardial infarction (Kaplan, 1982; Pepys et al.,1981). The current review of CRP will cover all its aspects with particular consideration for its role in the inflammatory response and subsequently with its specific relationship to current C-reactive protein studies in periodontal diseases.

1.7.2 Biosynthesis of C-reactive protein

Experimental studies of CRP biosynthesis in the rabbit have been pre-assumed to reflect CRP biosynthesis in man. Two sites for CRP synthesis have been reported, and these are hepatic and extra hepatic sites.

1.7.2.1 Hepatic origin of CRP synthesis

The only cells in the liver responsible for CRP synthesis are the hepatocytes. This was first reported by Hurlimann et al.(1966) who used isolated liver cell cultures, and has been supported by Riley et al.(1969), Kushner and Feldman, (1978), and also by Kushner et al.(1980) who used isolated

perfused livers and identified the presence of CRP in the cytoplasms of the hepatocytes of inflamed rabbits where CRP-producing cells were recruited centripetally from the portal zone. The recruitment starts in the periportal areas and then spreads to involve all cells across the liver lobule during the inflammatory process (Kushner and Feldman, 1978).

It has been shown by light microscopic studies that hepatocytes contained CRP during the acute phase response while ultrastructural studies confirmed that these cells are the site of origin of this protein which is localised to rough endoplasmic reticulum, Golgi apparatus and smooth endoplasmic reticulum (Kushner and Feldman, 1978; Kushner et al.,1980). Studies have indicated that, CRP which is found in serum and in damaged tissue, is formed by conversion of circulating precursors to pentameric CRP at sites of tissue injury or at other sites distant from the liver (Kushner and Kaplan, 1961; Kushner et al., 1963). All the above studies indicated that the liver is the source of CRP in serum and in inflammatory lesions and that tissue injury results in the induction of CRP synthesis in hepatocytes. They also indicated that the capacity to synthesize CRP does not appear to be limited to a subpopulation of hepatocytes because when maximally stimulated, virtually all hepatocytes appear capable of synthesizing CRP.

1.7.2.2 Extrahepatic origin of CRP synthesis

There is no evidence for CRP production by any organ other than the liver except from a few investigators (Ikuta et al., 1986; Kuta and Baum, 1985,1986), who demonstrated that a subset of peripheral blood lymphocytes (PBL) which exhibit natural killer activity are able to synthesize CRP, which apparently remains bound to the surface of these cells and is called surface CRP(S-CRP). This S-CRP is not acquired exogeneously. Kuta et al.(1986) and Baum et al.(1983) studies showed that CRP expressed by PBL is a membrane protein that does not appeared to be secreted. Murphy et al.(1991) demonstrated that human peripheral blood mononuclear cell (PBMC) express the CRP gene and based on the studies by Kuta et al.(1986), concluded that human PBMC can synthesized CRP. This is the first report on the extrahepatic transcription of human CRP. They also concluded that hepatocytes may synthesize and release pentameric CRP which when protected from denaturation remains inactive until it reaches the site of inflammation, where the lymphocytes may synthesize and express the conformating active form.

1.7.3 Secretion, metabolism and kinetics of CRP synthesis

CRP dramatically increases in concentration in the blood of many animals in response to inflammatory stimuli or tissue

destruction and the termination of the process is followed by a return of CRP levels to normal. CRP is produced by progressively increasing numbers of hepatocytes after an inflammatory response and secreted into the serum (Kushner and Feldman, 1978). Continuous increase in the rate of CRP synthesis appears to be dependent on continuing exposure to extrahepatic factors (Kushner et al., 1980).

Rates of CRP synthesis in vitro are more directly related to the duration of the inflammatory response in vivo and the serum CRP level at the time that the liver was isolated in the experimental animals. The post-inflammatory elimination of CRP is equally rapid with the half life estimated at approximally 8.9 hours (Kushner et al, 1980). The in vivo half life of CRP in the circulation of rabbits is 4-6 hours and is the same in normal and stimulated animals, the rapid rate of catabolism reflecting a rapid rate of synthesis relative to blood levels (Chelladuria et al., 1982).

The functional catabolic rate is the same in stimulated and unstimulated animals and the amount of CRP catabolized per day is thus proportional to the plasma concentration (Pepys et al., 1983). The kinetics of human CRP response are compatible with a rate of synthesis and turnover comparable to those seen with rabbit CRP (Pepys et al., 1983).

Studies of human CRP response showed that the peak level of CRP which is attained, as in the case of myocardial infarction, is correlated with the severity of tissue damage (Kushner et and Feldman, 1978), and reaches its peak

in 2 or 3 days and then comes down fairly quickly. The level depends on the duration of increased CRP production rather than upon the initial rate of rise (Kushner and Feldman, 1978).

The experimental studies suggested that a great degree of tissue injury results in more protracted formation of the postulated CRP-inducing mediator. This leads to continued maximal CRP synthesis by the liver for a longer period, with consequent attainment of higher serum CRP levels. It is inferred from these studies that the striking and rapid changes in serum CRP concentration reflect a continuing rise in the rate of hepatic CRP synthesis, and that CRP is newly synthesized and rapidly secreted.

Once the induction of CRP synthesis by hepatocytes is initiated the induction process involves the progressive recruitment of increasing numbers of hepatocytes to CRP synthesis. More extensive tissue damage results in prolonging the induction of CRP synthesis and consequently in higher serum CRP levels and this may be due to more protracted activation of the mediator system. After a variable period of time following stimulus, again related to the extent of tissue injury, serum CRP levels usually fall sharply, presumably reflecting sudden diminution in hepatic synthesis of CRP (Kushner and Volanakis, 1982).

The secretion of CRP is increased during the course of the acute phase response, and is regulated either by alteration in the availability of specific receptors on the hepatocyte membrane or by competition between different secretory

proteins for common receptors. The secretion by the liver varies over 1000-fold range depending on the presence and severity of inflammatory stimuli (Macintyre et al., 1985). Studies on rabbit CRP showed that the hepatocytes retard the secretion of CRP and this inhibition diminishes as the acute phase response progresses. The relationship between the change in synthetic rate and efficiency of secretion is not a causal one, but both synthesis and secretion are regulated by independent intracellular mechanisms during the acute phase response (Shou-Ih Hu et al., 1988).

1.7.4 CRP STRUCTURE

CRP belongs to a family of proteins known as pentraxins, a name derived from their unique molecular configuration of polypeptide subunits arranged in groups of five in a form of disks with cyclic pentameric symmetry (Pepys et al., 1985). CRP, complement component C1t and serum amyloid P component (SAP) are all related to the family of pentraxins (Pepys et al., 1985). They are phylogenetically ancient, have a unique structure and a calcium-dependent ligand binding specificity for phosphorylcholine. CRP structure resembles both the C1t component and SAP in amino acids sequence homology and appearance under electron microscope (Osmand et al., 1977). The pentameric form of the CRP molecule has been confirmed by the rotation function studies of two tetragonal crystal forms of human CRP (Myles et al., 1991).

Early studies by Gotschlich and Edelman (1967) showed that the CRP molecule has a pentramic form consisting of an aggregate of identical six subunits each having a m.w.of 21,000 daltons corresponding to a total m.w. of 129,000, daltons held together by non-covalent interactions.

The primary structure of CRP has however been determined by protein sequencing (Oliveira et al.1979), and consists of five identical single polypeptide subunits with molecular weight ranging from 21,000-23,000 daltons; each of these subunits is noncovalently linked and assembled in annular disk-like configuration with a cyclic pentameric symmetry with m.w. ranging from 110,000-to 140,000 daltons for the intact molecule. The subunit structure consists of a single polypeptide chain of 187 a.a. with disulfide bounds. Each subunit, within the intact molecule, binds one or two calcium ions (Gotschlich and Eldman, 1967).

Robey et al. (1987) found that the primary structure of human CRP revealed three regions evenly distributed through out the protein. Each of these regions contains peptide sequence closely resembling the amino acid sequence of the immunomodulator peptide tuftsin that is found in the CH_2 domain of the Fc segment of the immnoglobulin (Ig) heavy chain. They concluded that human CRP contains three peptides resembling tuftsin.

The human CRP gene together with serum amyloid P component gene is located on the proximal long arm of chromosome 1 (Floyed-Smith et al., 1986; Sehgal et al., 1989).

1.7.5 FORMS OF CRP

CRP has been reported to exist in two forms: native CRP which electrophoreses as a gammaglobulin, and fast-CRP (F-CRP) which electrophoreses as an alpha globulin and which expresses, upon urea chelation and acid treatment or heating, an antigenic reactivity form which is distinctive from that of native CRP (Potempa et al., 1983a). The antigen expressed on F-CRP could be expressed on free subunits (pre-CRP) of human CRP (Mantzourians et al., 1983).

Potempa et al.(1987) reported that F-CRP represents free human CRP subunits, and they introduced the term "Neo-CRP" to describe the antigenicity associated with free CRP subunits. Potempa et al.(1983a,1987,1988) confirmed that CRP can be structurally modified by heat, acid, or urea chelation to express a neoantigen. Potempa et al.(1988) confirmed that the neo-CRP antigen is not only expressed by the modified CRP which remains soluble (free CRP subunits), but it could be also expressed on the aggregated fractions. CRP complexed with CPS in the fluid phase does not express the neo-CRP antigen.

It has been found that under certain adsorption or modification conditions, CRP undergoes conformational alterations leading to the release of CRP subunits from the pentamer, these subunits having unique surface characteristics hallmarked by the appearance of neo-CRP (Potempa et al., 1983; Potempa et al.1987).

Potempa et al.(1987) also reported that the neo-CRP molecule has a m.w. of approximately 22,000 daltons, and the CRP neo-antigen can be expressed on free CRP subunits without proteolysis. They further reported that the molecules expressing the neo-CRP antigens are present in the acute phase plasma as in the case of rheumatoid arthritis.

Neo-CRP has been reported to be naturally expressed in tissues at sites of inflammation and necrosis (Zeller et al., 1986), and on the surfaces of a subpopulation of human PBL expressing large-granular lymphocyte morphology and, interestingly on B lymphocytes (Bray et al., 1988).

CRP subunits may arise in situ by de novo synthesis as well as by native-CRP dissociation. The release of free subunits in vivo may occur in the areas of acute inflammation where hydrophobic sites become exposed on lipoproteins or necrotic membranes. The forms of CRP expressing neo-CRP epitope have the ability to activate and/or modulate the functional responses of platelets, polymorphs and monocytes(Potempa et al., 1988).

Studies by Shan-Ching et al.(1989) using monoclonal antibodies, demonstrated five epitopes on the native CRP molecule and three on neo-CRP, with one of the epitopes expressed on native-CRP being retained on the modified forms of the molecule.

Since CRP forms expressing the neo-CRP antigen binds to and stimulate macrophage and neutrophil responses and are even more protective than native CRP against lethal pneumococcal

infections, this neo-antigen may well serve as an important marker for relevant biological functions of CRP.

1.7.6 Serum CRP Levels

For a certain period, following its discovery in 1930 due to the invalidity and insensitivity of the semiquantitative techniques used in its detection in serum, CRP was recognized as the protype acute phase protein associated with pathological conditions only and not present in sera from healthy subjects.

The methods used by early investigators (Wood et al., 1951; Nilsson et al., 1968) reported serum CRP simply as absent or present or elevated. Such misinterpretation has limited its clinical application as a disease marker. Now, due to the development of reliable, valid and sensitive quantitative techniques CRP has been appreciated as normally present as a trace constituent of plasma in all healthy subjects. The clinical measurement of serum CRP is valuable as a screening test for a number of organic diseases and as a sensitive objective index of disease activity or response to therapy in some inflammatory, infective and ischaemic conditions (Kaplan, 1982; Pepys et al., 1981).

CRP is exceptional among human acute phase proteins to be present at low level in normal healthy subjects (less than 0.1 mg/L),and in the rate and extent of its elevation(up to 3000-fold) in response to acute phase stimuli (Kushner and

Volanakis, 1982). CRP rises markedly in concentration in association with inflammation and tissue necrosis, and it has been found deposited at sites of cell injury (Kushner and Volanakis, 1982).

1.7.6.1 CRP levels in healthy subjects

CRP was first reported to be present in sera from healthy subjects by Seligman et al.(1959) in trace amounts, and this was confirmed by Goldwasser et al.(1961). Clause et al.(1976) and Kindmark (1972) demonstrated the presence of CRP in all normal sera in very small amounts, and their results indicate that CRP is a quantitative, and not a qualitative phenomenon. Clause et al.(1976) using sensitive radioimmunoassays detected CRP to levels of 3 mg/l. They also reported that the levels increase rapidly up to 1,000-fold within 24-48 hours reaching concentrations as high as 300-500 mg/l during intense inflammatory reactions. They concluded that all individuals normally have small amounts of serum CRP with a median level of approximately 0.6 mg/l and a normal range of 0.07-8.20 mg/l.

Shine et al.(1981) confirmed CRP to be present in trace amounts in the serum of healthy individuals with a median value in healthy adults of 0.8 mg/l. They found that the level of serum CRP in 90% of individuals was less than 3 mg/l while 99% had less than 10 mg/l. They also reported that CRP is universally present in serum.

Other studies found that most apparently healthy

individuals have serum CRP levels less than 0.2 mg/l, but levels as high as 1mg/l are not infrequently found (Drahovsky et al., 1981). Levels below 1.0 mg/l have being considered as insignificant elevations.

Recently, Shapiro et al.(1989) have drawn a reference range of 0.05-4.0 mg/l at 95% confidence interval and up to 10.0 mg at 99% confidence interval,for serum CRP in healthy adults.

1.7.6.2 Serum CRP levels in diseases

It is evident that a great range of elevated serum CRP levels above those found in apparently healthy individuals may be seen in disease. The sera with significant elevations of CRP are divided arbitrarily into two groups, moderate elevation (1-10 mg/l) or marked elevation (greater than 10 mg/l) (Drohorsky et al 1981). CRP levels rise in serum within hours of the onset of inflammation, reaching a peak during the acute stage and decreasing with the resolution of inflammation or trauma (Pepys and Baltz, 1983).

In response to most forms of injury, infection,inflammation , or tissue damage the serum CRP level increases rapidly and dramatically up to 1000-fold its normal level. It can reach levels as high as 300-400 mg/l within 48 hours of an acute stimulus, and persist at high levels in individuals with significant bacterial infection or active non-infective inflammatory disease (Pepys and Baltz, 1983;

Kushner and Volanakis, 1982).

CRP increases in serum in association with many different types of inflammation and in response to a variety of stimuli. Most commonly, it is related to bacterial infections, ischaemic injury to tissue as in myocardial infarction, malignant neoplasms associated with tissue necrosis, physical or traumatic injury such as bone fracture, surgery or burns, and inflammatory conditions such as rheumatic fever, rheumatoid arthritis, vasculitis, and ulcerative colitis (Kaplan 1982). It has been suggested that the amount of CRP response varies directly with the degree of tissue injury, with the type of inflammatory response, and with the organ or tissue involved in the inflammation. CRP responses occur more frequently in bacterial than in viral infections (Kaplan 1982). Quantitative studies on human CRP responses have revealed that there are highly significant differences between the levels attained in response to different diseases (Pepys 1982) which may be due in part to the underlying pathological process. Although the detection of elevated levels of serum CRP is not specific for any particular disease, it is a useful indicator of inflammation.

1.7.7 Binding specificities of CRP

1.7.7.1 Binding at the sites of inflammation

CRP has been detected on the surfaces of necrotic cells at

sites of inflammation (Kushner and Kaplan, 1961; Kushner et al., 1963) and in tissue damaged in several naturally occurring or experimentally-induced pathological conditions. Kushner and Kaplan (1961) using direct immunofluorescence reported CRP deposition in necrotic skeletal muscle fibers of the rabbit, with a selective localization to the inflammatory site and absence from cells from non-inflamed tissues and organs. CRP was detected in necrotic myofibers in rabbits after an inflammatory stimulus, but not before its appearance in the blood. Kushner et al.(1963) reported the deposition of CRP in damaged myocardium. Tissue localization or deposition of CRP is reported as selectively associated with acute but not chronic inflammation (DuClose et al., 1981).

There are several reports of CRP deposition <u>in vivo</u> in human inflammatory conditions. Parish (1977) found deposition of CRP in cutaneous lesions of patients with vasculitis. Gitlin et al.(1977) reported CRP localization in rheumatoid arthritis patients. It has been suggested that the binding of CRP to necrotic or altered cells may reflect binding to altered membrane structures,in which phosphotidylcholine, sphingomyelin, or both were exposed. In this way it may activate the classical pathway of the complement system, thus inducing cell lysis and releasing complement mediators for chemotaxis and removal of necrotic cell debris (Volanakis 1982; Kushner and Volanakis, 1982).

1.7.7.2 CRP binding sites

Phosphorylcholine-containing ligands of CRP, polyanions and polycations are all integral constituents of cells and likely to be abnormally exposed in or released from damaged tissues. CRP could function in plasma as a recognition mechanism for abnormal materials of both extrinsic and intrinsic origin via its interactions (Pepys, 1982). CRP does not interact with the intact membranes of healthy normal cells in vivo or in vitro.

CRP has two distinct but interactive ligand binding sites. The first one is a calcium-dependent site which binds to phosphorylcholine residues (Volanakis and Kaplan, 1971), but can also bind other ligands which lack phosphoryl choline such as certain bacterial polysaccharides (Heidelberger et al.,1972) lipids, heparin, nucleic acids and dextran sulphate (Gotschlich et al.,1982). This binding specificity of CRP is directed toward phosphorylcholine (pc) and choline phosphatides. The best ligand for the CRP molecule is phosphorylcholine (Volanakis and Kaplan 1971) but it also binds other phosphate mono-esters (Gostchlich, , 1982) and non-phosphate or choline-containing polysaccharides (Heidelberger et al., 1972). CRP has a capacity for calcium-dependent binding to phosphorylcholine in C-polysaccharides of pneumococci (Kushner et al.1963; Gostchlich 1982). The affinity of CRP for phosphorylcholine is much greater than for other phosphate monoesters, and choline itself cannot bind CRP (Gostchlich et al.1982).

The second binding site for CRP does not require calcium ions and forms complexes with polycations, including poly-L-lysine, poly-L-arginine, lysine-and arginine-rich histons, myelin basic protein and leukocyte cationic protein (Siegal et al., 1974; DiCamelli et al., 1980; Potempa et al., 1981), as well as with positively charged non phosphorylcholine-containing liposomes (Tsujimoto et al. 1980). The poly cation-binding site may be a part of the phosphorylcholine-binding site or at least be allosterically modulated by it.

1.7.7.3 CRP binding reactivities

CRP has binding reactivities with various materials.

1.7.7.3.1 Calcium and non calcium-dependent binding

Generally two major groups of binding reactivities have been reported for CRP.

A.The first group is a calcium-dependent reaction of CRP with CPS, which has been found to be largely dependent on specific reactivity with phosphorylcholine groups (Volanakis and Kaplan, 1971), or as a calcium-dependent binding with carbohydrate polymers of galactose (Gewurz, 1982) and galactosamine.

1.Calcium-dependent binding with carbohydrates:

CRP interacts in vitro with D-galactose-containing poly saccharides, including polysaccharides containing galactosamine (Higginbotham et al., 1970), and certain polysaccharides similar to Nacetylgalactosamine (Oliveria

et al., 1980).

2.Calcium-dependent binding with phosphorylcholine groups: Studies by Volanakis et al.(1971) showed that CRP can bind directly to phosphate monoesters in presence of calcium ions, and they suggested that choline phosphate present in CPS may provide the major reacting site of CPS with CRP. The phosphorylcholines are found in cell walls of bacteria as CPS in pneumococci and in other bacteria as well as on the surfaces of fungi and parasites (Gewurz, 1982). They are also present but are not exposed in human cell membranes as a part of phosphatidyl choline (Gewurz, 1982). When human cell membranes are disrupted through injury or inflammation, their phosphorylcholine groups are exposed and provide CRP with a potential binding site (Gewurz, 1982).

The functional effects of CRP binding reactivity with phosphorylcholine-binding site are that in vitro it can activate complement (Kaplan and Volanakis, 1974) and enhance phagocytosis (Ganrot and Kindmark, 1969; Mortensen et al, 1976). In vivo it protects mice from lethal infection with Streptococcus pneumoniae (Mold et al., 1982). Studies by Nakayama et al.(1984) showed that CRP has a selectively inhibitory effect on the antibody response to phosphorylcholines.

B.The second group of binding reactivities for CRP is calcium-independent binding and involves polycations including myelin basic protein and leukocyte cationic proteins (Dicamelli et al., 1980; Siegel et al., 1975). CRP

also shows binding specificity to certain polyanions (heparin, hyaluronic acid), to other polycations (protamine sulphate and histone,L-lysine,L-arginine), and to chromatin and nucleic acids.

Certain polycations, including protamine sulfate, polymers of L-lysine and leukocyte cationic proteins, could activate the complement system via their reactivity with CRP (Siegel et al., 1975). The concept of a binding reactivity for polycations is supported by the observations that CRP binds and reacts with positively charged liposomes, causing activation of the complement system (Richard et al., 1979). Studies by Potempa et al.(1981) showed that in the presence of phosphorylcholine (PC), CRP rapidly precipitated and formed complexes with polycationic polymers, suggesting a relationship between CRP-polycation binding site and the sites for calcium and PC. Their data are consistent with Oliveira et al.(1980), who reported a 2-locus binding site on CRP, one requiring calcium and is responsible for phosphorylester binding, and the other not requiring calcium and responsible for the binding of the cationic group of phosphocholines.

Studies by Potempa et al.(1983) indicated that CRP-poly cation interactions are significantly and selectively influenced in the presence of small amounts of polyanions such as heparin, chondroitin sulfate, and hyaluronic acid but not DNA. Interactions of CRP with the above groups of ligands may result in activation of the complement system, and may have a physiologic significance during the acute

inflammatory process.

1.7.7.3.2 Binding of CRP to Liposomes

Liposomes consist of concentric lamellae of lipid bilayer membranes. Generally liposomes are composed of phospholipids such as lecithin or sphingomyelin but they also may have other lipid consistuents such as cholesterol, charged lipids, and glycolipids. Liposomes can be prepared with a marker such as glucose trapped in the aqueous interspaces that separate the concentric membranes (Richards et al., 1977). Studies of binding of CRP to liposome model membranes have demonstrated the above two categories of binding sites and have shown that complement consumption and membrane lysis can result from these reactions (Volanakis et al., 1979).

Studies by Richards et al.(1977,1979) indicated that CRP interactions with liposomes in human serum result in consumption of hemolytic complement,complement-dependent membrane damage and release of trapped marker (glucose) from the liposomes. This reaction requires a positively charged membrane and is influenced by other variations in membrane composition.

1.7.3.3 Antibodies

Studies by Ballou et al.(1990), using purified human CRP, were unable to detect clinically important binding of CRP to human IgG; monoclonal IgG1 (monomeric IgG), polyclonal

IgG, heat-aggregated IgG, experimentally prepared immune complexes (ICs) or ICs from human serum samples. They concluded that native human CRP does not have a clinically significant binding capacity for immunoglobulin, and the previously reported presence of CRP in ICs may result from, or be facilitated by an association with components other than immunoglobulin such as antigens or complement components.

1.7.3.4 Fibronectin

Salonenee et al.(1984) reported that immobilized CRP binds soluble plasma fibronectin, but not in the soluble phase. Immobilized CRP also was found to bind effectively to fibrinogen. Fibrinogen competes with fibronectin for CRP binding sites. They suggest that the CRP deposits may trap at the site of injury the circulating fibronectin, and concluded that CRP possibly acts as a fibronectin receptor in damaged cells. Their results provide a link between acute phase response and tissue repair.

1.7.3.5 Chromatin (Cr) and Nuclei (Nucleic acids, Nucleosome core particles)

Chromatin is composed of equal amounts of DNA and histons, which are organized into repeating subunits known as nucleosomes (Duclos et al.1991).

CRP binds to several nuclear constituents including small nuclear ribonucleoproteins (snRNP) (Duclos et al.1990).

Studies by Robey et el.(1984) showed that CRP binds to chromatin and chromatin fragments which may be present in the extracellular milieu at sites of tissue damage. This binding in the presence of complement could lead to chromatin degradation into nucleosome-sized fragments.

Shepherd et al.(1986) reported binding of human and rat CRP to their respective autologous liver nuclei. This binding is mediated by a CAP polycation site.

Studies by Duclos et al.(1991) reported CRP binding to native sub-nucleosomal and chromatin fragments, and demonstrated chromatin binding to histones H_2A-H_2B diamer and (H_3-H_4)tetramer. They concluded that CRP binds to nucleosome core particles, and their results indicate that CRP binding sites include H_1 and part of other histons in chromatin.

The functional effects of CRP binding to chromatin:

Binding causes chromatin solubilization and degradation (Robey et al., 1984). It enhances micrococcal nuclear digestion when bound to nuclei, and this enhancement indicates that CRP binding could alter the chromatin structure and leads to increase in the rate of chromatin digestion.

CRP may act as a scavenger of chromatin fragments released from damaged cells, and may help mediate the removal of chromatin and/or chromatin fragments from the body after cell death provided the barriers separating CRP from chromatin are destroyed (Robey et al., 1984). Thus CRP may play an important role in the recognition and clearance of

nuclear material from damaged cells (Robey et al., 1984). Duclose et al.(1990) reported that CRP is rapidly and effectively transported into the nucleolus of mammalian cells. Their studies also showed significant similarities between CRP and nucleoplasmin in nuclear localization activity. They concluded that one possible effect of CRP localization would be an alteration of RNA processing and initiation of the degradation process of nuclear chromatin of damaged cells to fragments resembling nucleosomes. Thus, CRP may play a unique role in injured cells to alter processing of damaged nuclei.

1.7.8 CRP interactions with cellular elements of immune system, and complement system

CRP interacts with the cellular elements of inflammation ,including neutrophils (polymorphs), monocytes/macrophages, lymphocytes, and platelets, as well as with complement system.

1.7.8.1 CRP interactions with Neutrophils (Polymorphs)

Many studies have been conducted on the interaction of CRP with polymorphs (PMN). CRP interacts with neutrophils in vivo and in vitro (Shephard et al., 1986; Zeller et al., 1986; Muller et al., 1986; Butch a et al., 1987a).
In general native CRP alone is ineffective in stimulating human PMN (Shephard et al., 1986; Zeller et al.,1986). However under certain conditions, some forms of complexed or aggregated CRP both bind to and modify biological activities of human PMN (Shephard et al., 1986; Kilpatric and Volanakis, 1985; Muller et a., 1986).
The biological activities modified, include stimulation of motility (Wood, 1951; Butcha et al.; 1987b), enhancement of attachment to endothelium (Muller et al.1986), phagocytosis (Kindmark,1971;Morstein et al., 1977; Garnot and Kindmark , 1969), and superoxide anion production (Dobrinch and Spagnulo, 1991).

a.Binding:

Several studies have shown that PMN possess a specific cell

surface receptor for CRP, which may be either associated with, or identical with, IgG Fc gamma receptor, in as much as aggregated IgG can act as a competitive inhibitor of CRP binding (Muller et al., 1986; Shephard et al., 1986; Kilpatrick and Volanakis, 1985; Zeller et al.1986,Butcha et al.1987b Shaphard et al., 1986; Dobrinch and Spagnuolo 1991; Ballou et al., 1989). The presence of specific binding sites for CRP on neutrophils supports the concept of CRP acting as a nonspecific opsonin.

b.Phagocytosis:

CRP has been shown in vitro to have an influence on PMN phagocytosis. Aggregated CRP at high concentrations activates complement, thereby attracting PMN and inducing release of lysosomal enzymes (Parish, 1977).

Studies have reported that native CRP complexed with pneumococcal C-polysaccharide (Pn-CPS) has an opsonic activity for human PMN phagocytosis. In these studies it was found that the opsonic activity is dependent on both the ability of CRP to activate complement and the presence of phosphorylcholine in the bacterial capsule [Mold et al., 1982; Edwards et al., 1982; Kindmark, 1971; Garnot and Kindmark, 1969; Mortensen et al., 1976).

Kilpatrick and Volanakis (1985) showed that stimulated neutrophils can be induced to phagocytose Pn-CPS-CRP complex, and that both Pn-CPS and CRP-mediated interactions with the neutrophils are necessary for generation of the phagocytic signal. They concluded that native CRP function is to variously enhance phagocytosis by acting as an

opsonin. Shephard et al.(1986) concluded that the uptake of CRP and CRP-CPS complexes into neutrophils appears to be via a specific CRP-dependent mechanism, independent of complement activation.

Klipatrick et al.(1987) demonstrated that lymphocytes release a low m.w.soluble factor(s) or a modulator(s) that induce(s) phagocytosis of CRP-coated cells by activated PMN. This phenomenon occurred under conditions similar to those in vivo.

The above studies and those which demonstrated binding of soluble CRP to PMN (Muller et al., 1986; Butcha et al. , 1987b; Bullou et al., 1989) support the primary opsonic role for CRP and suggest that the major immuno-modulatory function of CRP is enhancement of phagocyte clearance mechanisms. So it could be concluded that CRP acts as an opsonin for PMN and has a biological property in its ability to recognize foreign pathogens and initiate their elimination by interaction with the complement system and stimulated PMNLs.

C.PMN response to CRP:

Migration, adherence and chemotaxis:

Muller et al.(1986) showed that CRP interaction with PMN IgG Fc receptors mediates the adherence of the PMN and this may be important in the localization of the inflammatory focus.

Wood (1951) showed that soluble CRP stimulates the directed migration of PMN. Native CRP has been shown either to inhibit chemotaxis (Butcha et al., 1987a),or to have an

influence on chemotaxis (Robey et al., 1987; Shephard et al., 1986).

CRP can inhibit neutrophil chemotaxis in vitro at high concentrations when it may control the inflammatory response by down-regulating neutrophil migration(Kew et al., 1988,1990).

Chemiluminescence (CL):

Zeller et al.(1986) found that aggregated CRP (but not native CRP) binds to and selectively modulates the response of PMN to IgG Fc receptor-induced respiratory burst activity measured by chemiluminescence.

The studies by Potempa et al.(1988) showed that neo-CRP consistently, and in a dose-dependent manner, potentiated the respiratory burst response of human PMN to heat-modified IgG. Conversely Tatsumi et al.(1988) found that platelet activating factor (PAF)-induced chemiluminescence of neutrophils was suppressed by native CRP.

Superoxide anion production:

Dobrinch and Spagnuolo (1991) demonstrated that purified human CRP impairs the oxidative capacity of neutrophils (PMN) by two mechanisms. One mechanism is through interaction with receptors on neutrophils causing direct cell inhibition, while the other is by direct antioxidant activity causing scavenging of superoxide anion. CRP may thus have a regulatory role in oxygen-radical production during the inflammatory response in a dose dependent manner.

Relatively high levels of purified native CRP can inhibit

superoxide anion generation in vitro (Kew et al., 1986). Butcha et al.(1987a) reported a dose dependence of neutrophil response to CRP. An initial stimulation of PMN function at low CRP concentrations, which includes enhancement of directed migration of PMN with maximal effects at 1mg/l has been found, with slightly potentiated activation of oxygen generation and secretion by certain agents at concentrations up to 5 mg/l. With increasing concentrations of CRP (above 10 mg/l) an inhibition of superoxide anion production, chemotaxis and vitamin B12-binding protein secretion was observed.

The kinetics and dose dependence of the neutrophil response to CRP appear to be ideal for a physiological inhibitor of the potential tissue toxicity resulting from neutrophil functions. A low level of CRP could allow full expression of such functions with maximum damage to the infecting agent but with increasing CRP levels,'super-activation' of phagocytes would be prevented, thereby minimizing damage to the host. Thus CRP may function as a negative feedback mechanism for the potentially cytotoxic neutrophil functions, limiting phagocytic oxidative mechanisms, and down-regulating the inflammation.

Butcha et al.(1988) found that human CRP causes a dose-dependent decrease in the extent of activation of superoxide production, granule secretion, and protein phosphorylation in stimulated neutrophils. Binding of CRP to PMN appears to play a dual role. At concentrations of 1mg/l or less CRP elicits PMN chemotaxis and phagocytosis.

At higher concentrations (>10mg/l) it may inhibit oxygen radical generation, superoxide production and secretion, chemotaxis, and protein phosphorylation (Butcha et al.1988; Butcha et al.1987a].

Neutrophil (polymorphs) response to the peptides of degraded CRP:

Degradation of CRP by neutrophils may result in release of peptide fragments, whose effect may be different to that of native CRP (Robey et al., 1986).

Robey et al.(1987) found that in vitro native CRP did not induce phagocytic leukocytes either to chemotax or to produce superoxide. Proteolysis or degradation of purified CRP in vitro by human neutrophil-derived acid proteases produced peptides with potent immunoregulatory properties on leukocyte function, similar to those of tuftsin molecules. These properties include stimulating phagocytic cells to chemotax, the production of superoxide, and induction of mononuclear cells to produce IL-1 at concentrations similar to those required for tuftsin to also induce these phenomena.

Shephard et al.(1988) found that CRP intralysosomally processed by enzymes, resulted in soluble peptides of m.w. <14000 dalton which at a concentration of 2mg/l modulate neutrophil functions, including inhibition of chemotaxis, superoxide production, degranulation and phagocytosis.

The studies of Shephard et al.(1989) indicated that CRP-derived peptides at an inflammatory site resulted from CRP degradation by neutral serine protease of the membranes of

PMA-stimulated neutrophils. These peptides can inhibit many of the proinflammatory functions of PMN such as superoxide production,and can oppose the tissue destructive potential of these cells. They concluded that CRP functions as an up regulatable substrate for membrane-associated neutrophil serine protease(s).

Shephard et al.(1990) reported that small soluble peptides are produced in vitro when CRP is degraded with non-stimulated or PMA-stimulated PMN and purified neutrophil membranes. They demonstrated that three of these peptides (CRP-III,CRP-IV,CRP-V) significantly inhibit chemotaxis, and superoxide production from activated neutrophils at high concentration. They concluded that CRP serves as a dramatic upregulatable substrate yielding bioactive peptides on degradation that oppose the tissue destructive potential of activated PMN.

The above studies together with those of Dobrinch and Spagnuolo (1991) support the notion that CRP may have a regulatory role in oxygen-radical production during acute inflammation in a dose dependent manner.

CRP peptides: based on the observation that the primary structure of human CRP contained three regions with peptide sequences closely resembling the amino acid sequence of the phagocytic stimulator tuftsin (Robey et al.1987), it was proposed that CRP acts as a substrate for phagocyte derived enzymes which liberate bioactive peptides at the sites of inflammation(Robey et al., 1987; Shephard et al., 1988). Peptides generated when CRP is degraded by neutrophil

lysosomal enzymes (Robey et al., 1987; Shephard et al., 1988), modulate certain aspects of the acute phase response. Peptides generated in this way were shown to inhibit various classical neutrophil functions (Shephard et al., 1989; Robey et al., 1987; Shephard et al., 1988) and inhibit platelet aggregation (Fiedal et al., 1988). Cleaved forms of CRP, containing the tuftsin sequence Thr-Lys-Pro-Arg, either enhance (Robey et al., 1987), or inhibit (Butcha et al., 1986) PMN chemotaxis, lysosomal enzyme release, and superoxide anion generation.

1.7.8.2 CRP interactions with Monocytes/Macrophages

a.Binding:

Several investigations in vitro have showed that CRP has a specific binding site on monocytes/macrophages (Kearney et al., 1982; Whisler et al., 1983; Mortensen, 1982; Zeller et al., 1986; Honda et al., 1987). Murine and human monocytes/ macrophages also express membrane CRP (MCRP) receptors (Zahedi et al., 1988; Zeller et al., 1989). The MCRP occurs as a membrane-associated protein expressed on liver when it helps in galactose-specific binding of certain ligands (Kempaka et al., 1990).

Studies indicate that CRP binds to human monocytes at a site physically associated with but distinct from IgG Fc receptors, and CRP-reactivity may be influenced by an association with this receptor (Zahedi et al., 1988; Zeller et al., 1989; Ballou et al., 1989; Tebo and Moretensen , 1990; Ballou and Cleveland, 1990). CRP is generally assumed to be produced and secreted by hepatocytes and presumably the liver macrophages acquire CRP as secondary phenomenon.

b.Phagocytosis:

CRP has been shown to have an influence on phagocytic activity of monocytes/macrophages. The presence of CRP on particle surfaces has been reported to facilitate their ingestion by human monocytes(Mortensen et al, 1976), and mouse macrophages (Mortensen and Duszkiewicz, 1977). Mortensen et al.(1977) showed that the presence of both CRP and cleaved-complement components (C3b,C4b) on the

particles as erythrocyte membranes was necessary for the efficient attachment and uptake by the macrophages. CRP complexes bind to murine human macrophages and in the presence of complement can be ingested by these cells in vitro.

c.Monocytes/Macrophages response to CRP:

Aggregated or complexed CRP with its ligand can interact with human monocytes (Zeller et al., 1986b; Zeller et al., 1986a; Janco et al.,1983).

Inhibition of precursor cell recruitment:

Marcelletti et al.(1982) reported that high CRP concentrations specifically inhibited the in vitro clonal proliferation of normal granulocyte-macrophage precursors which possess IgG Fc-receptors and are committed to the mononuclear phagocyte lineage. CRP has no effect on macrophage progenitor cells stimulated by an inflammatory response.

Mortensen (1983) reported that CRP selectively inhibited in vitro normal monocyte colony formation of granulocyte-monocyte colony forming cells derived from bone marrow . The results are consistent with a regulatory role for CRP during monocytopoiesis induced by inflammation.

The above studies suggest that CRP functions as a negative feedback regulator of the cellular composition of the acute phase of an inflammatory response.

Immunomodulatory effect of CRP on monocytes/macrophages:

CRP in a monomeric form, complexed with its ligands or aggregated has been shown to act as a immunomodulator of

macrophage/monocyte function.

PAF-induced activation of monocytes has been shown to be inhibited by CRP in vitro (Tatsumi et al., 1988). CRP has also been shown to have migration inhibition factor (MIF)-like activity (Whisler et al., 1986). Barna et al.(1984) demonstrated that liposome-associated CRP is readily endocytosed by macrophages and enhances both superoxide anion production and tumoricidal activity of macrophages. Zahedi and Mortensen (1986) found that purified CRP activated inflammatory mouse macrophages to a tumoricidal state independently of both lipopolysaccharides and lymphocytes.

Barbara et al.(1987) found that purified human CRP activated monocyte-mediated cytotoxicity and oxidative metabolism, in a dose-dependent manner when exposed to acute phase serum levels of CRP in vitro. So CRP may serve as a biologically relevant macrophage activator/ monocyte modulator contributing to the nonspecific host response associated with the early stage of the acute inflammatory response.

Zeller et al.(1986a) demonstrated that aggregated-CRP selectively augments Fc receptor-mediated stimulation of monocyte oxidative metabolism, the respiratory burst activity being measured by chemiluminescence. They suggested a significant role for CRP in the modulation of monocyte activity at the site of inflammation.

Honda et al.(1987) suggested that the initial CRP binding to monocytes and the subsequent internalization initiate

the synthesis of IL-1.

Whisler et al.(1986) showed that low concentrations (but not high) of CRP (2.5-10 mg/l) increased monocyte chemotaxis. CRP alone is sufficient to result in some increase in procoagulant activity (PCA), and causes further increase in PCA in the presence of CPS and immune complexes. CRP plays a role in modulating the chemotaxis and (PCA) of monocytes during the early stages of inflammation.

Barna et al.(1988) found that CRP at high level induced a dose-dependent cytotoxicity in human alveolar macrophages in vitro. This supports the previous studies by Barna et al.(1984), which showed an enhancement of tumoricidal activity in macrophages in experimental animals in vivo and in vitro, and in human monocytes exposed to CRP in vitro. Robey et al.(1987) found that in vitro native CRP did not induce macrophages/monocytes to chemotax or to produce superoxide. Proteolysis or degradation in vitro of purified CRP by human neutrophil-derived acid proteases resulted in peptides with potent immunoregulatory effects, similar to those of tuftsin molecules. The effects include stimulating monocytes to chemotax, production of superoxide, and induction of mononuclear cells to produce IL-1 at concentrations similar to those required for tuftsin to induce similar phenomena.

Activation of monocytes/macrophages significantly augmented by proteolytic fragments of CRP (Robey et al., 1987; Shephard et al., 1988, 1989, 1990). The proteolytic

fragments activate macrophages in a similar way to tuftsin or substance P (Robey et al., 1987; Shephard et al., 1988, 1989,1990) with stimulation of monocyte phagocytosis, chemotaxis, superoxide and IL-1 production (Garnot and Kindmark, 1968). Such activating fragments may be generated early in infective diseases by proteases released from necrotic cells including granulocytes or from the infective agents. The fragments could also be generated within macrophages during endocytosis or phagocytosis of ligands opsonized either by S-CRP or by their MCRP, thereby connecting the opsonic activity with activation of the immune system.

1.7.8.3 CRP interactions with Lymphocytes

a.Binding:

Interaction of CRP with lymphoid cells:

Purified CRP alone does not detectably bind to lymphocytes (James et al.1981a), but in the presence of an appropriate ligand such as pneumococcal CPS or phosphocholine it can bind to normal lymphocytes, particularly those with the IgG Fc receptor. Binding is increased in the presence of acute phase sera such as occurs in rheumatoid arthritis and streptococcal infections (William et al., 1980). The studies of James et al.(1981b) indicated that CRP-binding occurs with T cells, B cells, and null cells. The studies also indicated that CRP-binding cells predominantly represent a subset of cells bearing Fc receptor, and these cells have the morphological characteristics of large granular lymphocytes. Subsequently James et al.(1983) demonstrated the presence of S-CRP on the surfaces of lymphocytes. Studies have indicated the presence of CRP antigenicity on the surface of lymphocytes involved in the mediation of natural killer activity ((Kushner and Volanakis, 1982) .

Kuta and Baum (1986) suggest that some lymphocytes, the large granulated lymphocytes (LGL), produce small amounts of CRP themselves and express it on their cell surfaces, the surface CRP (S-CRP) not being acquired exogenously from trace levels of CRP in normal serum. The lymphocytes that produce S-CRP in this way apparently do not secrete CRP into serum or tissue fluids.

Ikuta et al.(1986) reported the synthesis and secretion of CRP by peripheral mononuclear cells in culture, under the stimulation of a factor derived from activated monocytes. They suggest that this factor is relevant to metabolites of an arachidonate cascade.

The findings of Samberg et al. (1988) indicate that a molecule identical to a neo-antigenic form of CRP is present on the surface of a significant proportion of normal human lymphocytes. They also demonstrated the preferential expression of neo-CRP, and not native-CRP,epitopes on these cells.

Bray et al.(1988) demonstrated that a neo-antigenic form of CRP, or a cross-reactive molecule, is present on the surface of natural killer cells and B cells, and suggest that this molecule (neo-CRP) may be physically associated with Fc gamma receptors present on these cells.

CRP effects on Lymphocytes:

Binding of CRP to T-lymphocytes can result in modulation of their functional characteristics in vitro. Hornung et al. (1971) showed that low levels of purified CRP stimulated lymphocyte blastogenesis, while Hokama et al.(1973) found inhibition of phytohaemagglutinin (PHA)-stimulated blastogenesis. They suggest that serum CRP is responsible for the depression of the lymphoblastogenic response to mitogens that is observed in many disease processes. Mortensen (1975) reported that CRP binds selectively to T-lymphocytes (TL), inhibits their ability to form spontaneous rosettes with sheep erythrocytes, and also

inhibits their response to allogenic cells in mixed lymphocyte culture (MLC) reactions. CRP was effective in inhibition of blastogenesis in undirectional MLC reactions. CRP failed to inhibit phytohaemagglutinin (PHA) or concavalin A (Con-A)-induced mitogenesis.

Mortensen (1975) also reported that CRP does not bind to B lymphocytes (BL) nor does it alter their functions such as their ability to activate complement components (C3b), binding to a portion of Ig (IgG), or the mediation of antibody-dependent cytotoxicity (ABDC) reactions.

Mortensen and Gewurz (1976) reported that CRP bound to murine T lymphocytes, inhibited mixed lymphocyte reaction and the generation in vitro of cytotoxic T-cells.

Mortensen et al.(1979) reported that human CRP, added to murine cultures, inhibited in vitro induction of T lymphocyte-dependent IgM and IgG antibody formation, by a CRP-mediated generation of a suppressive T-cell population. CRP may thus have an immunoregulatory function that limits the cellular response during inflammation.

Crofts et al.(1976) found that CRP binds to lymphoblasts formed upon stimulation with antigens but not with mitogens. Collectively CRP reacted selectively with a subpopulation of T-lymphocytes and influenced certain T-cell-dependent functions.

Mortensen (1976) showed that purified human CRP inhibits antigen-induced lymphocyte proliferation, chemotactic factor (lymphokine) production and both antigen- and mitogen (PHA, Concavalin A)-induced MIF (macrophage

inhibition factor) formation. CRP decreased the production of MIF and Migration chemotactic factor (MCF) from both antigen and mitogen-stimulated lymphocytes (Mortensen et al., 1977). The studies of Mortensen (1977) indicated that CRP mediates the suppression of antibody induction to T-dependent, but not T cell-independent, antigens by interaction with T cells and thus generating the suppressor T-cell population. The results demonstrated CRP-mediated inhibition of the antibody plaque forming cells response to T-dependent but not T-independent antigens.This inhibition required the presence of CRP-binding T-cells.

Mortensen (1982) found that the pokeweed mitogen (PWM)-induced generation of polyclonal-secreting cells was inhibited,in a dose-dependent manner by both purified human CRP and CRP-CPS complexes. CRP and CRP-CPS complexes mediated suppression by binding and activating monocytes and T cells with IgG Fc receptor. These findings indicate that CRP has the potential to modulate antibody response during the course of an inflammatory response.

CRP enhances cell-mediated cytotoxicity at pathological concentrations in a dose-dependent manner (Veter et al., 1983), and in vitro CRP and CRP-CPS complexes significantly enhance cell-mediated cytotoxicity, minimally enhanced the mixed lymphocyte reaction, and induce a small but regularly detectable blastogenic response in resting PBL.

The studies of Whisler et al.(1983) showed that CRP considerably augments the early development of B-cell colonies.This augmentation is maximal during the initial

stages of colony formation. Thus relatively modest concentrations of CRP can promote focal B cell proliferative responses, and so one of the biological functions of CRP is the ability to modulate the activities of the B cell system.

Wangel and Kontiainen (1983) suggested that high levels of CRP induce human peripheral blood mononuclear cells to elaborate a suppressor factors(s) with an effect in vitro on polyclonal B cell activation. The factor suppresses the plaque forming cell response in three major Ig classes in a similar manner to that of Concavalin A generated suppressor factor in respect to its time of action, its effect on the Ig classes M, G and A, its lack of cytotoxicity and some structural aspects.

Whilser et al.(1986) found that CRP inhibited the promotion of B-cell colony formation by autoactivated T cells. CRP directly reduced T4 helper cell promotion of colony formation rather than inducing T8-mediated suppression of the T4 cells. Their studies demonstrated that CRP can directly reduce the ability of autoreactive T4 cells to expand the B-cell compartment, and block T-cell activation events proximal to proliferation.

Vetter et al.(1986) concluded that the most significant effect of CRP on lymphocytes is a substantial increase in cell-mediated cytotoxicity. CRP enhancement of the killing generated in a mixed lymphocyte culture is due to enhancement of the cytotoxic T-cell response. This enhancement may serve both to increase the specific

cytotoxic T-lymphocyte (CTL) response and to broaden the specificity of the in vivo cell-mediated cytotoxicity response, since physiological concentrations of CRP are elevated 24-48 hours after infection.

CRP and Natural Killer (NK) Activity:

The results of Baum et al.(1983) demonstrated that CRP is present on certain NK effectors, and that the cell-surface CRP and not the fluid-phase CRP is involved in the mediation of NK activity, suggesting that the involvement is at the level of the effector cell.

Baum et al.(1987) suggest that although CRP appears to be involved in NK-mediated killing, it is not involved in effector target cell-mediated recognition. Their studies confirmed the previous studies that S-CRP is involved in NK responses.

Hamoudi and Baum (1991) provided evidence which indicates that anti-CRP blocks NK-mediated lysis at the calcium dependent stage of lysis. Events that follow this stage in the lytic process are also inhibited. They reported that this inhibition occurs during a calcium-dependent stage that immediately follows conjugate formation. This provides direct evidence that CRP involvement is a post-binding event.

1.7.8.4 CRP interactions with the complement system

Activation of the complement (C) system by CRP was first demonstrated by Kaplan and Volanakis (1974), who reported complement interaction with CRP complexes such as those with pneumococcal C-polysaccharide, choline phosphatides, lecithin and sphingomyelin. This activation induced complement consumption with marked depletion of the early acting complement components C1, C2, C4, and to a lesser degree complement components C3-C9 via the classical pathway independent of the presence of specific Ig. The reaction resulted in electrophoretic conversion of complement component C3, indicating that an effective C3-convertase had been formed. Interaction of CRP with lipid emulsions containing cholesterol and other phospholipids also resulted in classical pathway activation of complement.

Segial et al.(1974) found that the polycation protamine sulphate induces consumption of complement in acute phase serum by interaction with CRP, and this interaction involves activation of the primary classical pathway at the level of complement component C1.

Volanakis and Kaplan(1974) reported that the presence of complement C1q is a selective requirement for complement activation by CRP complexes.

Claus et al.(1977a) reported interaction of CRP with complement Cb at the level of complement Cq1 in a similar way to immunoglobulin. Studies by Jaing et al.(1991)

indicate that a conformation-determined region on CRP binds to a sequence-determined region on the collagen-like region (CLR) of C1q leading to complement activation.

Segial et al.(1975) reported that CRP interaction with polycations such as poly-L-lysine polymers and other naturally occurring polycations such as leukocyte cationic protein, arginin-rich histones, and myelin basic protein resulted in complement activation with depletion of complement components C1 to C5 as well as early complement components. Osmand et al.(1975) found that CRP initiated complement-dependent passive haemolysis of cell coated C-polysaccharides. It was subsequently shown that CRP interaction with erythrocyte-pneumococcal-CPS and serum or complement components resulted in the deposition of fragments of C3b and C4b on the cells which could mediate the adherence of such cells to both B lymphocytes and monocytes expressing receptors for complement components. This resulted in enhanced phagocytosis by monocytes (Mortensen et al., 1976). This mediation by CRP of reaction, adherence and phagocytosis by monocytes and B-cells points to the significant role of CRP in host defence.

CRP may augment phagocytosis by two distinct routes, serving as an opsonin and possibly increasing the activation of phagocytic cells (Mortensen et al., 1976). Claus et al.(1977b) reported that CRP significantly enhanced the activation of complement by salt-like polyanion-polycation complexes. Examples of polyanions are

heparin, DNA, hyaluronic acid, chromatin sulfate, and dextran sulfate while examples of polycations are protamine sulfate and poly-L-lysine.

Volanakis and Narkates (1981) reported that liposomes with a particular lipid composition bind CRP in the membrane leading to classical complement pathway activation, and the lipsome-bound CRP could then bind human C1q,the recognition protein of the classical pathway.

Miyazawa and Inoue (1990) found that even in the absence of phosphocholine-containing or polycation-containing ligands, CRP can cause complement activation in mildly acidic conditions, which are within the physiological range normally found at an inflammatory locus.

The previous studies indicated that CRP-initiated complement activation can proceed to opsonization, and suggested that these reactions may occur in vivo since CRP has been demonstrated to be deposited on damaged tissue at sites of inflammation. CRP may thus cause complement cleavage product generation at inflammatory sites and also mediate the subsequent clearance of damaged cells or cellular constituents from these sites.

Volanakis.(1982) showed that CRP-C-polysaccharide precipitates can be solubilized by the action of human complement, the reaction resulting in the deposition of complement proteins on both CRP and pneumococci. Their studies presented preliminary evidence for covalent binding of C3 fragments to CRP and pneumococci.

Edwards et al.(1982) reported that CRP greatly enhanced the

complement-mediated chemiluminescence (CL) response of PMN to pneumococci type 27 in normal serum and in serum lacking Ig. These findings support the concept that CRP plays a protective role in bacterial infection.

Berman et al.(1986) showed that CRP-complement classical pathway activation is restricted to the early parts of complement activation (C1, C4, C2, and to lesser extent C3) but does not involve significant consumption of complement components C5-C9 (membrane complex attack) and so complement-mediated lysis does not occur. They concluded that CRP binding to nucleated cells leads to complement activation without cytolysis as no activation of membrane complex attack occurs.

Mold and Gewurz (1981) showed that CRP in contrast to Ig, inhibits alternative pathway activation by either pneumococci, streptococci or liposomes. CRP binding to membrane or bacterial surfaces can convert them from alternative pathway activation to classical pathway mechanisms. Mold et al.(1984) showed that CRP inhibition of the pneumococcal activation of complement alternative pathway is due to increasing the interaction between factor H and C3b on pneumococcal surfaces, which causes inhibition of C5 converatase activity of both classical and alternative pathways. This phenomenon may limit the complement reaction beyond C3, and may prevent damage by C3-C9 at the sites of inflammation while at the same time allowing effective opsonization. Either type of CRP binding,i.e.calcium-dependent binding to phosphocholine-

containing pneumococci, and calcium-independent binding to polycation liposomes, could result in both classical pathway activation and alternative pathway inhibition.

Generally once CRP bound to phosphocholine-containing cell walls (necrotic or damaged but not normal cells), aggregated by heat and has reacted with phosphorylcholine -containing or cation-containing ligands or reacted with polycation-polyanion complexes, it can activate the classical complement pathway effectively (Kaplan and Volanakis, 1974; Volanakis and Kaplan, 1974; Siegel et al., 1975, Osmand et al., 1975; Claus et al., 1977a,1977b). This activation occurs by C1q and C1 activation, and does so as effectively as IgG antibody.

All the functional and biological effects of complement activation are generated by CRP, including opsonization by fixation of C4b and C3b and by lysis (Mortensen et al., 1976; Mold et al., 1982, Osmand et al.1976). When CRP binds to some of its ligands, including positively charged liposomes and whole pneumococci, it inhibits activation of the complement alternative pathway and initiates instead activation by the classical pathway (Mold and Gewurz, 1981; Mold et al., 1984), which involves the attachment of CRP to the activating surface.

CRP reactivity with complement and effects on phagocytosis:

CRP may function in host defence. In the presence of complement, CRP appears to have opsonic properties. The reactivity of CRP with a variety of substances(e.g. microbial products, damaged tissues) released or exposed as

a result of inflammation, or in association with model membranes, and cationic molecules, has been shown to result in activation of the classical pathway (Edwards et al., 1982). This leads to complement-dependent phagocytosis by a process for which the presence of CRP is required. In the case of human CRP, there is no doubt that complement activation can then generate chemotactic factors and the opsonic fragments of fixed complement proteins. CRP and complement were found to stimulate ingestion of erythrocytes by human monocytes or mouse macrophages in vitro (Mortensen et al., 1976, Mortensen et al., 1977),and to alter clearance patterns in vivo (Nakayama et al., 1982).

The biological importance of CRP interaction with complement indicates the importance of complement activation products in the elimination of foreign antigens. The functions of CRP may relate to its ability to specifically recognize cellular elements carrying phosphocholine which is available for reaction and this may then initiate their elimination by interaction with complement system.

Studies in vivo have demonstrated that CRP can activate the classical pathway of complement after complexing with certain polyanions or pneumococcal capsular C-polysaccharide antigen (Kaplan and Volanakis, 1976), which then enhances opsonization (Edwards et al., 1982).

There is evidence from in vitro studies with monocytes that, following complement fixation by CRP binding to a

particular ligand, the presence of both CRP and fixed complement is required for internalization (Mortensen et al., 1976; Mortensen and Duskiewicz, 1977). Mortensen et al (1977) found that enhanced phagocytosis was dependent on both the presence of CRP and the deposition of fragments of C3 and C4 on the indicator cells. Mortensen et al.(1977, 1976), Claus et al.(1977a,b) and Edwards et al.(1982) reported that enhancement of phagocytosis by mouse macrophages was mediated through a direct interaction of CRP with macrophage Fc receptors which activate the classical complement pathway and enhance opsonization. Comparable results have been obtained in vivo in mice (Mold et al., 1982).

1.7.8.5 CRP interactions with Platelets

Many investigations have been carried out on CRP interaction with platelets, and controversial results obtained.

Fiedal and Gewurz (1976a) found that CRP, in a dose-dependent relation, causes inhibition of the aggregation of human platelets stimulated by modified human Ig (heat-aggregated) or thrombin. It also inhibits the activation of platelet factor 3 by the same stimulants.

Fiedal and Gewurz (1976b) found that CRP causes inhibition of platelet reactivities stimulated by poly-L-lysine, adenosine diphosphate (ADP) and epinephrine in platelet-rich plasma (PRP). It also inhibits the platelet aggregation stimulated by collagen in PRP. CRP inhibits the activation of platelet factor 3 (PF3) and the release of serotonin during stimulation of platelets by ADP, and arachidonate.

Marder et al.(1977) demonstrated that higher concentrations of both rabbit and human CRP are capable of inhibiting the aggregation of rabbit platelets and the retraction of a fibrin clot.

Fiedal et al.(1977) reported that CRP inhibits the release of platelet adenosine diphosphate (ADP) and/or serotonin at the same time as inhibition of platelet aggregation stimulated by aggregated human IgG occur.

Simpson et al.(1982) reported that activation of platelets by modified heated-CRP (H-CRP) results in the secretion of

both platelet dense bodies and an alpha-granule constituent. The results also showed the formation of thromboxane B2.

Fiedal et al.(1982a) reported that the platelet inhibitory effects of CRP preparations, previously reported as inhibition of platelet activation resulted from association with low molecular weight factor.

Fiedal et al.(1982b) reported that modified CRP (H-CRP),and CRP when complexed with polycations (poly-L-lysine, and protamine) but not with C-polysaccharides, induced the reactions of aggregation and secretion from isolated platelets. This generated a platelet release reaction with aggregation and secretion occurring coincidentally. Pure isolated, native CRP does not affect platelet function (Fiedel et al., 1982a,b). The results of Fiedal et al(1982c) indicate that the platelet receptors for the modified forms of CRP and IgG are distinct.

Fiedal (1985) reported that the reversible aggregation and secretion of ATP by platelet-rich plasma activated by ATP, became irreversible due to the presence of H-CRP. Aggregated CRP appears not to be an agonist in PRP but only a strong synergist, synergizing with known platelet agonists in plasma, and not acting as a direct platelet activator itself. The studies indicated that the platelet-directed activity associated with CRP is not utilized unless platelets are otherwise challenged.

Fiedal et al.(1986) reported that cleaved forms of pentameric CRP resulting in certain CRP peptide fragments

can inhibit platelet activation and aggregation. CRP in a degradative environment, such as at sites of inflammation /tissue damage or through the action of serum protease, may thus temporarily down-regulate the platelet function.

Vigo (1985) reported that CRP inhibits platelet aggregation induced by PAF and can stabilize platelet membranes against the lytic effects of lysophosphatidyl choline. Inhibition of platelet aggregation is accompanied by an inhibition of arachidonic acid release from both phosphatidylcholine and phosphotidylinostitol. CRP binding to membranes can protect them against the detergent-like effects of lysolipids and from the action of phospholipases.

Inhibition of phospholipases by CRP results in an inhibition of arachidonic acid release, thereby blocking the production of inflammatory mediators. CRP might thus act as an early protective recognition mechanism in acute inflammation. By binding to membranes, CRP prevents further tissue destruction and stabilizes cellular membranes against detergent-like effects.

The study by Fiedal (1988) suggests that aggregated CRP initially stimulates platelet activation independent of fibrinogen but in doing so activates a fibrinogen-dependent mechanism capable of augmenting the overall extent of platelet stimulation.

Fiedal and Gamble (1988) concluded that aggregated CRP and ligand-complexed forms of CRP initiate the activation of platelets, whereas naturally occurring CRP peptides inhibit platelet activation. Their studies reported that neutral

proteases of PMN cleave CRP into reactive products (peptides) with the potential to inhibit platelet activation. They found that a minimum of two linear functional domains exist in the CRP molecule located in the mid-portion and C-terminus, and which influence platelet activation. The mid-portion domain inhibits platelet activation stimulated by ADP or acid-soluble collagen, whereas the C-terminal domain initiates platelet activation. Both the CRP domains contain a homologue of the immunoregulatory signal peptide tuftsin.

Janos et al.(1991) reported that CRP (1-20mg/l) inhibited PAF-induced aggregation of human platelets in a time and dose-dependent manner.

So CRP may be an important modulator of platelet activation during the acute inflammatory reaction. In general many of the reports on the effect of CRP on platelets are controversial. A self-complexed form of CRP and CRP aggregated with heat or modified with oxygen species were shown to amplify platelet activation by ADP, epinephrine, thrombin and collagen (Fiedal, 1985; Miyazawa et al., 1988). Augmentation of PAF-induced aggregation also has been reported (Kohakayama et al.1986). In contrast, native pentameric CRP has been found to inhibit platelet activation (Fiedal et al., 1976a,b).

The elevation of CRP levels during inflammation and tissue destruction thus may serve to limit platelet functions.

1.7.9 Biological functions of C-reactive protein

CRP provides one of the several signals that trigger and/or regulate immune surveillance by augmentation or inhibition of various components of the immune response. This is an essential and beneficial function of CRP.

CRP provides a synergistically acting signal only, a help which often facilitates homeostasis and which appears in some instances to be an essential factor for homeostasis to occur. Measurement of CRP may be used as an indicator of the acute phase response. The measurement of levels of CRP in serum, cerebrospinal fluid or urine are widely used for rapid and early diagnosis of various inflammatory conditions and to monitor the course of inflammation. Elevation of plasma levels of CRP during the acute phase of an infection is generally regarded as a favorable prognostic indicator.

Despite a large number of investigations, the biological function(s) of CRP and its role in the inflammatory response are still not fully clear. in vitro studies have shown that CRP potentially has both pro- and anti-inflammatory properties. The precise in vivo function of CRP has not been elucidated even though its remarkable phylogenetic conservation suggests an important beneficial role during inflammation.

The stable conservation of the existence of CRP throughout vertebrate evolution, including its molecular structure and ligand-binding specificity suggest that it has an important

biological role which is presumably beneficial to the organism.

The main role of CRP may be to provide for enhanced clearance of inappropriate materials from the plasma whether these are of extrinsic origin, such as microorganisms and their products, or of intrinsic origin such as autologous products of cell damage and death.

In general the biological functions of CRP include the following:

1.Ligand binding:

The specific and broad spectrum ligand-binding reactivity of CRP in all species enables it to recognize products of a wide range of different microorganisms. Binding to these products may reduce their toxicity or role in pathogenicity , while involvement of secondary processes such as complement activation and phagocytosis may also contribute to protection against infection. The efficacy of this function, at least for human CRP in mice infected with pneumococci, has been clearly demonstrated (Mold et al., 1981; Mold et al., 1982a). Patterson et al.(1968) concluded that CRP participitates in non specific resistance to bacterial infection in mice and rabbits, and thus it may function as an opsonin or bacteriolytic agent. This supports the concept that CRP may function both in lower animals, which have not evolved specific antibody mechanisms, as well as in higher animals when it may function at an early stage of infection before production of specific antibody gets underway.

2.Opsonization:

A potent opsonic role has been proposed as a major CRP function in man (Kilpatric and Volanakis, 1985; Mortensen et al., 1977), and this is supported by evidence of specific binding receptors for CRP on human phagocytic cells, including neutrophils and monocytes.

Investigations of opsonic properties of CRP resulted from the initial finding of CRP binding to Streptococcus pneumoniae and a number of other bacteria (Kindmark, 1971; Mold et al.1982). Kindmark (1971) reported that CRP stimulates the phagocytosis of various species of pathogenic bacteria, including Gram-positive and Gram-negative pathogens. CRP may combine with bacteria to act as an opsonin with consequent promotion of non-specific resistance to infection.

Human CRP which has been aggregated or complexed with multivalent ligand is a potent activator of the classical complement pathway (Volanakis, 1982; Volanakis and Narkates , 1983), and by virtue of this effect it can act as a complement-dependent opsonin in vitro (Mortensen et al., 1976). Many investigators have speculated that CRP may function in vivo by opsonizing damaged and necrotic tissue for subsequent phagocytosis (Pepys, 1981; Pepys and Baltz, 1983; Pepys et al., 1985).

3.Resolution and repair of damaged tissue:

CRP has been demonstrated in rabbits in association with necrotic muscle fibers in induced ischaemic lesions of the myocardium (kushner and Kaplan, 1961; kushner et al.,

1963), and in man CRP has been reported in the lesions of cutaneous vasculitis (Parish, 1977) and in association with synovial cell nuclei in biopsies from patients with rheumatoid arthritis (Gitlin et al., 1977).

Human CRP does not bind to the intact membranes of healthy living cells, or to artificial membranes which mimic their composition or structure. It does however bind to damaged or altered cell walls and to artificial phospholipid membranes, and having done so it then activates complement (Volanakis , 1982). It has been therefore suggested by Volanakis that the role of CRP may be to recognize damaged cells and their products. It is thought that CRP regularly binds to damaged cells in vivo ,and by then activating complement to generate the chemotactic and opsonic activities required to promote phagocytosis, leads to resolution and repair of the lesion (Kushner and volanakis, 1982).

4.Clearance of abnormal materials from the circulation:

It has been suggested that a function of CRP is to promote the clearance of abnormal materials from the circulation and/or contribute to their detoxification (Pepys, 1981, Lancet). Experiments have shown that CRP does indeed mediate or enhance blood clearance of pneumococcal C-polysaccharide-coated erythrocytes (Kilpatrick et al., 1987; Kilpatrick and Volanakis, 1985; Mold et al., 1981). , and this also requires an intact complement system.

Increased production of CRP is stimulated as effectively by autologous damage in the absence of infection as it is by

infection. During the course of tissue damage a variety of cellular constituents may get access to the plasma.

If an abnormal material of extrinsic origin,such as a microorganism or microbial product, is in the plasma it too may have its clearance enhanced by CRP provided that it bears appropriate ligand(s). This function of CRP has been demonstrated with pneumococci and with C polysaccharide-coated erythrocytes. Human CRP is able to activate a normal mouse complement system, and initiate opsonization and phagocytosis in a similar manner to immunoglobulin. CRP may therefore have a role in organ sequestration if CRP binds to the component cells (Nakayama et al., 1982).

The interaction between aggregated CRP and plasma low density lipoproteins (LDLP) may result in the clearance of LDLP from the circulation and so have a role in the pathogenesis of atherosclerosis (Pepys and Baltz, 1983).

5.Inflammation:

Intradermal or intracutaneous injection of C-polysaccharides in patients with acute pneumococcal pneumonia or with rheumatic fever, elicits an immediate wheal and flare reaction which resolves over an hour or so and which is then followed by a delayed reaction (Francis and Abernethy, 1934; Finland and Dowing, 1935). The lesion is oedematous and erythematous,and may have a central haemorrhagic area. In normal healthy subjects, or convalescent patients whose serum CRP levels have fallen toward normal, injection of C-polysaccharides has no

effect. Injection in experimental animals of aggregated CRP which had previously been incubated with human complement and had fixed C1q, elicited similar lesions with neutrophil infiltration and fibrinoid necrosis (Parish, 1977). These findings suggest the possibility of this mechanism initiating inflammation or tissue-damaging process when circulating CRP levels are high. In vivo CRP has been shown to be deposited at sites of ischemia and necrosis (Kushner et al., 1961; kushner et al., 1963), and several studies have shown that CRP is deposited almost exclusively in neutrophil-rich inflammatory lesions, whereas little or no CRP is observed in lesions composed primarily of mononuclear cells (Duclos et al., 1981; Parish , 1977; Gitlin et al.,1977).

CRP may be particularly important during the early periods of inflammation when the antibody titres are low. CRP meets the criteria of a physiological response modifier since its concentration in blood, as well as in tissues, changes dramatically during the early course of the inflammatory response.

One function of CRP within an inflammatory site is to clear the inflammatory focus, including bacterial clearance and allow tissue repair to proceed (Nakayama et al., 1983). Since CRP is known to be deposited at the site of tissue destruction, it may be expected to exert its influence on the macrophages infiltrating these sites.

While it is possible that CRP may be enhancing inflammation and contributing to the chronicity of the lesions, it may

also be promoting healing. Yoshinori et al.(1989) concluded that CRP may play a critical role in the response to injury by serving as an opsonin for dead cells, in turn amplifying and focusing the inflammatory response on dead and damaged tissue, with both potential beneficial and pathological consequences. Monocyte ingestion of CRP-opsonized cells stimulates TNF-a secretion, which will further elevate CRP level. Thus, with tissue injury, cytokine generation and acute phase protein synthesis result in a system enhancing cellular debridement and promoting repair, perpetuated by the persistence of damaged tissue.

6.Interaction of CRP with cells from immune system

Many studies have been conducted on CRP-mediated effects on various inflammatory cells and immunocytes, notably the monocyte/macrophage and its precursor cell, the neutrophil polymorphonuclear leukocyte, the NK-cell and other lymphocyte subpopulation, and platelets.

CRP or CRP-derived peptides modify the behavior of all these types of cells. Studies on CRP interaction with these cells has resulted in a wide range of conflicting ideas , some of which are due to CRP existing in two different forms; the native pentameric molecule which is the major circulating form, and the neo-CRP which is a free CRP subunit exposing antigenic epitopes (Potempa et al., 1987). The activity of CRP may also depend on whether it is aggregated, complexed or cleaved.

Potempa et al.(1988) found Neo-CRP induces aggregation and secretions from platelets, and also potentiated platelet activation stimulated by ADP in platelet-rich plasma. Native CRP alone or when complexed with CPS does not cause induction. Forms of CRP expressing neo-CRP are not able to activate the C system, unlike CRP-CPS complexes.

In general native CRP alone is ineffective in stimulating human PMNL (Zeller et al., 1986; Shephard et al. ,1986), monocytes (Zeller et al., 1986; Potempa et al., 1988), platelets (Fiedal et al., 1982,1985), and lymphocytes (James et al., 1981,1982; Vetter et al., 1983). However under certain conditions, some forms of complexed or aggregated CRP both bind to and modify biological activities of human lymphocytes (James et al., 1981,1982; Vetter et al. , 1983), and platelets (Fiedal et al., 1982).

Platelets: platelets have specific receptors for CRP (Fiedal et al., 1982c), which inhibit aggregation (Fiedal and Gewurz , 1976a,b), but augment activation (Miyazawa et al.,1988). Aggregated CRP or complexed with its ligands stimulates platelet aggregation and release reactions (Fiedal et al., 1982).

Heated-CRP has been shown to induce aggregation and secretion in isolated platelets and to synergize with platelet activators to induce similar reactions in platelet-rich plasma (Fiedal et al., 1982; Fiedal et al., 1985)). CRP inhibits PAF-induced aggregation of platelets by inhibiting phospholipase activity and blocking the release of inflammatory mediators (Vigo, 1985; Fiedal et al

., 1982; Fiedal et al.,1985).

Neutrophils:

CRP binds to neutrophils (Shephard et al., 1986; Kilpatrick and Volanakis, 1985; Muller et a., 1986). Native CRP has been shown variously to enhance phagocytosis by acting as an opsonin (Kilpatrick and Volanakis, 1985), to inhibit superoxide anion generation, lysosomal release (Butcha et al., 1987), and chemiluminescence (Tatsumi et al, 1988; Zeller et al., 1986), and to influence on chemotaxis (Robey et al.,1987; Shephard et al, 1986). Heated-CRP has been shown to potentiate the aggregated IgG-induced respiratory burst activity of human neutrophils as measured by chemiluminescence (Zeller et al., 1986a,b).

Lymphocytes:

CRP augments T lymphocyte cell-mediated cytotoxicity (James et al., 1981,1982; Vetter et al., 1983,1986). It can both augment (Mortensen et al., 1982) and inhibits (Whilter et al., 1983,1986) B lymphocyte colony formation and inhibit B lymphocyte plaque formation (Vetter et al., 1986, Mortensen et al., 1982). CRP binds to natural killer (NK) cells as neo-CRP (Baum et al., 1983; Samberg et al., 1988) and is involved in NK cell activity at effector cell level but not at target recognition (Kempka et al., 1990).

Monocytes: CRP binds to human macrophages enhancing their respiratory burst response to aggregated IgG (Mortensen et al., 1976; Zeller et al., 1984) and mouse macrophages show a similar response (Zeller et al.;,1983; Potempa et al.,

1986; Mortensen et al., 1976). CRP augments monocyte activation and superoxide release (Zeller et al., 1986,1988; Potempa et al., 1988; Barna et al., 1988), but inhibits PAF-induced activation (Tatsumi et al. ,1986). It also inhibits MIF-like activity (Whiser et al., 1986), and precursor cell recruitment (Marcelletti et al., 1982; Mortensen et al., 1983), but it augments tumor cytotoxicity (Barna et al., 1984; Zahedi et al., 1986). Activation of monocytes/macrophages by proteolytic fragments of CRP is significantly augmented (Robey et al., 1987; Shephard et al., 1988,1989,1990). The proteolytic fragments activate macrophages in a similar way to tuftsin (Robey et al., 1987; Shephard et al., 1988,1989,1990). Such activating fragments may be generated early in infectious diseases by proteases released from dead cells or granulocytes or by the infective agents. They could also be generated within macrophages during endocytosis or phagocytosis of ligands opsonized by surface-CRP or via their membrane CRP. This connects the opsonic activity with activation of the immune system.

7.Phagocytosis and Complement Activation:

CRP complexed with CPS is a potent **in vitro** activator of the classical complement pathway (Gewurz et al., 1983;27; Kaplan and volanakis , 1974; Claus et al., 1977; Volanakis, 1982; Volanakis et al., 1983; Mold et al., 1982; Claus et al. , 1977), and enhances opsonization (Mortensen et al. , 1976; Edward et al., 1982; Holzer et al., 1984).

Studies in vivo have demonstrated that CRP can activate the classical pathway of complement after complexing with certain polyanions or capsular CPS antigen of pneumococcus (Kaplan and Volanakis, 1976; Edwards et al.,1982).

The reactivity of CRP with a variety of substances (microbial products, damaged tissues) released or exposed as a result of inflammation or the reaction with model membranes and cationic molecules, has been shown to result in activation of the classical pathway of complement (Edwards et al., 1982),and to complement-dependent phagocytosis.

In the case of human CRP, there is no doubt that this complement activation can then generate chemotactic factors and opsonic fragments of fixed complement proteins. There is evidence from in vitro studies with monocytes that, following complement fixation by CRP binding to a particular ligand, the presence of both the CRP and the fixed complement is required for internalization to occur (Mortensen et al., 1976; Mortensen and Duskiewicz, 1977). Mortensen et al.(1977) found that enhanced phagocytosis is dependent on both the presence of CRP and the deposition of fragments of C3 and C4 on the indicator cells, and the enhancement of phagocytosis by mouse macrophages was mediated through a direct interaction of CRP with macorphage Fc receptors.

1.7.10 CRP and Oral Diseases

There are few reports on the relation of CRP and oral diseases.

Adam and Christide (1962) reported a preliminary study of serum and saliva CRP levels and periodontal diseases, using a semiquantitative method (capillary precipitation test). They found that 50% of the patients were positive for both serum and saliva samples, primarily from cases of marginal periodontitis or periodontal abscesses.

Boucher et al.(1967) using a capillary precipitation procedure, tested sera for CRP from patients with various forms of inflammatory oral diseases (acute alveolar abscess, acute periodontitis, acute necrotizing ulcerative gingivitis, chronic marginal gingivitis) and from controls. The highest incidence of positive CRP tests and the strongest CRP reactions were observed in patients with acute alveolar abscesses. The CRP test became negative after antibiotic treatment and tooth extraction in two patients with acute alveolar abscess.

Shklair et al.(1968) used the slide-latex agglutination test to study the relation between the presence of serum CRP and periodontal diseases (severe and moderate cases of necrotizing ulcerative gingivitis (NUG), gingivitis, and periodontitis). The periodontitis group consisted of 10 cases of severe periodontitis which had generalized acute inflammation with much bone loss and pocket formation, and 8 cases of moderate periodontitis with only localized areas of bone loss and inflammation. They found that the presence

of CRP in the serum was more common in the severe than in the moderate periodontal group. When CRP was found to be initially positive, the CRP test became negative 3-7 days after the initiation of treatment in severe cases and a week following the initiation of treatment in moderate cases. This coincided with the elimination of the inflammatory stage. In the control group, 33 were negative for CRP. So in general patients with severe forms of periodontal disease usually develop CRP in their serum. The patients in the severe NUG group had the highest percentage of CRP production, followed by those with severe periodontitis, and then by severe gingivitis. The patients with moderate infections had a significantly lower incidence of CRP production. They concluded that a positive serum CRP test may be due to the presence of enough necrotic or injured tissue that can enter the blood stream to elicit a CRP reaction.

Iwamoto et al.(1979) using a latex particles agglutination test for the detection of CRP in human saliva, found that human whole saliva and sublingual-submandibular saliva was positive but parotid saliva was negative. The titre of salivary (supernatant) CRP showed a positive correlation with oral hygiene index of Green and Vermillion (1960), the debris index, the calculus index and the viscosity of saliva. Titres of CRP were significantly higher in whole saliva than in supernatant saliva. They concluded that the source of CRP in whole saliva is the sublingual-submandibular salivary glands and not the blood.

Adinolfi and Lehner (1976) studied the concentration of CRP and other acute phase proteins in sera from 40 patients with Behcet's syndrome and recurrent oral ulceration (ROU) using a single radial immunodiffusion technique. Their results showed significantly increased amounts of CRP in Behcet's syndrome. They concluded that CRP might be useful in the differential diagnosis of Behcet's syndrome, especially from recurrent oral ulceration. They suggested that during inflammation in recurrent oral ulceration, some of the acute phase proteins including CRP are increased. This increased CRP in some patients may modulate the immunological mechanism in such way, as by their binding to T lymphocytes, to promote phagocytosis and to activate complement. This induces a transition from focal oral ulceration to multifocal Behcet's syndrome. Lehner (1978) in his review stated that CRP could be involved in modulating the cell-mediated immune response by its effect on T lymphocytes. He concluded that at least two types of damaging immune mechanisms could be involved, the cell-mediated and the immune complex induced reactions, and these might account for the association between ROU and Behcets syndrome. Lehner and Adinolfi (1980) using radial immunodiffusion technique, showed that serum CRP increases significantly in all groups of Behcet's syndrome except the arthritis group, and does not increase significantly in cases of recurrent oral ulceration.

Martin et al.(1988) studied the levels of serum CRP and other acute phase proteins in sera of 36 patients with

chronic periapical granulomas before and after surgical treatment, using the rate nephelometry method. Mean serum concentrations of CRP were slightly increased at diagnosis, but had decreased significantly 3 months after apicectomy. The slight elevation of serum CRP may be due to the limited extent of the inflammation. The decrease of CRP levels reflect the complete recovery after elimination of local inflammation by apicectomy.

Aziz et al. (1990) carried out the first study of CRP in gingival crevicular fluid in a group of patients with chronic periodontitis and healthy control subjects using an ELISA technique. They also measured the level of CRP in saliva and serum in a cross sectional study. They were the first to report the presence of CRP in gingival crevicular fluid, and quantified its level in both the GCF and saliva. They concluded that CRP is present in the gingival crevicular fluid in both the patient and control groups.

Aziz et al. (1991) measured the level of CRP in serum and saliva in a group of patients with oral candidosis (denture stomatitis) before and after treatment using an ELISA technique, and found that both serum and salivary CRP levels were increased in oral candidosis and the levels fell in response to treatment.

Sibraa et al.(1991) used direct and indirect immunodot techniques in quantifying acute phase proteins including CRP in gingival crevicular fluid from periodontally diseased and healthy sites. Indirect immunodots were used to identify and establish relative amounts of CRP in two

diseased and two healthy sites in 24 periodontal disease patients. They found that CRP levels did not vary significantly between healthy and diseased sites. Their detection of CRP via indirect immunodot systems indicate its presence in both healthy and diseased sites, and their results supported the results of Aziz et al.(1990) on the presence of CRP in the gingival crevicular fluid.

Wannfors and Hansson (1991) studied the relation between serum CRP and other acute phase proteins in patients with acute osteomyelitis using electroimmunoassay. They found that CRP was markedly raised in four samples from three cases. The serum level also decreased rapidly when the initiating stimulus stopped. They suggested that the raised serum levels of CRP may be due to sample collection either during an intense initial phase or coincident with exacerbations. They concluded that a certain "mass of inflammation" seemed to be necessary before raised values of CRP were detected.

CHAPTER TWO

Clinical Materials and Methods

Aims, Subjects, Experimental Design, Clinical Methods and Data Analysis

2.1 Subjects

Two groups of subjects were selected for the study.

A.Control group:

This group consisted of twenty-five subjects with a mean age of 22.4+_1.5 years and a range of 20-26 years. The group contained 9 females with mean age of 22.7+_1.3 years, and 16 males with mean age of 22.2+_ 1.6 years. The subjects in this group were dental students in their final year at Edinburgh Dental Hospital. The selection criteria for this group were:

1.Each subject should be healthy with no history of any systemic disease or chronic infection at the time of the experimental study.

2.Each subject must not have been on steroids in the last six months before the start of the clinical study.

3.Each subject should have no history of periodontal disease, nor undergo any periodontal treatment before or during the study. He/she should have a healthy periodontal condition, with good oral hygiene and be on standard home mouth care.

B.Patient group:

This group consisted of 54 patients with a mean age 41.1+_9.2 years and a range of 20-60 years. Thirty five were females with a mean age of 37.5+_7.6 years, and 19 were males with a mean age of 47.7+_8.3 years. These were patients of the Department of Periodontology at Edinburgh Dental Hospital.

Selection criteria:

a.Inclusion criteria:

The subjects were selected on the basis that each of them should have a history of chronic periodontitis for at least one year before the start of the study. The history should include a presence of periodontal problems, including pockets of more than 4 mm already recorded with a PC12 probe (Prisma, UK) and kept in their standard periodontal pocket charts. Most of these patients were on maintenance therapy at one to three months recall, receiving normal prophylaxis which included scaling and polishing at most of their visits, and root planing on some visits. Treatment as described was continued for ethical reasons.

b.Exclusion criteria:

1.No history of serious systemic disease or recurrent chronic infection except periodontal disease.

2.No history of using steroids in the last six months before the study.

2.2 Selection of sites

Sites for both control and patient groups were selected according to the Technical clinical report series number 621 (Geneva WIIO 1979). WHO (CPITN index system) teeth and sites were selected from each subject's dentition for this study. These included, mesiobuccal sites for teeth number 16 (maxillary left first molar), 21 (maxillary left central incisor) and 24 (maxillary right first premolar). They also included the mesiolingual sites for teeth number 36

(mandibular left first molar), 41 (mandibular right central incisor) and 44 (mandibular right first premolar). If one of these sites was missing in the subject the next distal site was used or the next tooth in the dentition would be selected.

2.3 Aims of the study

2.3.1 General Aims

A.The development of a reproducible method of measuring the attachment level change. This aim was to measure small changes and reproduce accurate results. This measurement gave changes in the attachment levels (loss, gain or no change) among the sites and in both groups over one year.
B.Development of a reliable and valid method for the calibration of the Periotron 6000 to measure the volume of gingival crevicular fluid. This would be applicable to the wide range of the collected volumes of gingival crevicular fluid.
C.Development of a reproducible and sensitive technique to measure the levels of the acute phase protein, C-reactive protein (CRP) in serum, saliva and in particular in the small volumes of gingival crevicular fluid. This would be either by developing a new technique or establishing or modifying an existing technique.

2.3.2 Specific Aims

A.To investigate the presence of C-reactive protein in the saliva and gingival crevicular fluid from the selected sites in both control and patient groups, using the enzyme-linked immunoabsorbant assay (ELISA).

B.To investigate the possible association of C-reactive protein with the activity of periodontal disease measured by periodontal attachment loss, and also the possible predictive value of C-reactive protein in the destructive process of chronic periodontitis. This investigation includes the following objectives:

B1.The main objective is to correlate the clinical status of the subject using in particular the changes in attachment levels, with the levels of C-reactive protein particularly in gingival crevicular fluid collected from the related sites. This correlation is to be found sequentially at 2-monthly intervals over the observation period, and the over the one year period.

The objective is to detect clinically the disease activity indicated by loss of attachment at individual site or within each subject, with corresponding values of C-reactive protein.

B2.To seek any possible correlation between C-reactive protein and the following data:

a.Gingival inflammation using gingival index and bleeding on probing scores.

b.Pocket depth.

c.Oral hygiene using plaque index.

d.Microbiological composition of subgingival plaque using

the percentages of spirochaetes, motile rods,coccoid cells and other.

C.To seek any possible correlation between the clinical parameters and the disease activity as measured by attachment level loss.

D.To seek any possible correlation between subgingival flora, in particular spirochaetes and motile rods, with clinical parameters particularly with attachment level changes. The objective is to seek a possible correlation between loss of attachment and spirochaetes as well as motile rods.

2.4 Experimental design

1.A signed informal consent was taken voluntarily from each subject after a full explanation of the aims and methods of the clinical study.

2.Following their agreement to participate, an upper and lower rubber base impression was taken and from the resulting casts, a soft polythene stent for attachment level measurements was constructed for each arch. Subsequently, each subject was examined on six occasions over one year at 2 monthly intervals on average. No subject should have taken antibiotics for at least 72 hours before any examination date, otherwise the visit was postponed.

3.The following clinical data, measured as described subsequently were recorded at each visit for each subject

at the selected sites, and in the following order:

a.Unstimulated whole mixed saliva (at least 1ml) was collected at each visit by expectoration.

b.Plaque index scores were taken.

c.Gingival crevicular fluid from the selected sites at each visit, using a filter paper strip technique. The volumes were then measured using a Periotron 6000.

c.Gingival index scores were taken.

d.Probing pocket depth and attachment level measurements: These measurements were recorded twice at each visit. The first set of both pocket depth and attachment level measurements were recorded after the sites were sampled for GCF. During this period bleeding on probing (BOP) was also recorded. The second set of both pocket depth and attachment level measurements for all the sites were recorded after collection of the other clinical data in upper and lower jaw, starting again with the sites that were first sampled.

e.Subgingival plaque from the selected sites at each visit was collected immediately after the recording of the first set of attachment level measurements and the recording of BOP at each site.

f.Venous blood up to a volume of 10 mls was collected at each visit.

2.5 Clinical Methods

Only one examiner (SA), was used throughout these studies to record all the clinical parameters.

2.5.1 Plaque index (PI)

Plaque accumulation was measured according to plaque index of Silness and Loe (1964) which scored plaque accumulation on a scale 0 to 3 as follows:

Score 0 = tooth surface is clean, no plaque in the gingival area.

Score 1 = a film of plaque adhering to the free gingival margin and adjacent area of the tooth surface. The plaque can be removed only by running a sharp explorer across the tooth surface.

Score 2 = visible plaque on the tooth surface. Moderate accumulation of soft deposits within the gingival crevice ,on the gingival margin and/or adjacent tooth surface, which can be seen by naked eye.

Score 3 = abundant plaque within the gingival crevice and/or the gingival margin and adjacent tooth surface.

PI gave a separate recording for each of the selected sites and was called site PI scores, or the mean of the scores of all selected sites within each selected subject and this was called a subject mean for PI. The mean of the total scores for a group of teeth (sites) has been called mean site PI scores, and was used for the assessment.

2.5.2 Gingival index (GI)

The Gingival Index by Loe and Silness (1963) and Loe (1967)

has been used to assess gingival inflammation on each of the selected sites within each subject at each visit.

The following criteria have been used:

0 = absence of inflammation: clinically healthy gingiva.

1 = mild inflammation: there is a slight change in colour and little change in texture; no bleeding on pressure.

2 = moderate inflammation: there was moderate glazing, redness, Oedema, and hypertrophy; bleeding on pressure.

3 = severe inflammation: there was marked redness and hypertrophy; tendency to spontaneous bleeding; and ulceration.

GI gave a separate recording for each of the selected sites and was called single site GI scores, the mean of the scores of all selected sites within each selected subject has been used as a subject mean for GI. The mean of the total scores for a group of teeth (sites) was called mean site GI scores and was used for the assessment.

2.5.3 Bleeding on probing (BOP)

Bleeding on probing was used to detect the presence of gingival inflammation in particular at the base of the crevice or periodontal pocket. A pressure-sensitive probe (WHO pressure-controlled probe) set at 30 gram, with a blind ball point was inserted gently into the crevice at each selected site. Scoring was performed within 30 seconds of probing. Bleeding upon probing was scored dichotomously.

A score of 1 was given if there was bleeding while a score of 0 was given if there was no bleeding on probing.

2.5.4 Pocket depth (PD) and Attachment level measurement

A pressure-sensitive periodontal probe was made according to our instruction and need (Prima Instrument Company,U.K). The probe tip had a diameter of 0.6mm, with 1-13mm gradutions at 1mm increments, and deep marks at 4mm,7mm and 10mm depth. It is a modified type of hinge-controlled probe. The probe was calibrated by a calibrated screw to receive only 0.25N to 0.30N.

2.5.4.1 Development of a reproducible method for measuring attachment level changes and pocket depth (Modified stent technique)

A calibration trial was carried to develop a method for reproducibly measuring pocket depth (PD) and attachment level changes associated with periodontal disease, using a modified stent technique (Figure 2.1). This study compared the new technique with the conventional stent technique developed primarily by Isidor et al.(1984) in the measuring the pocket depth and attachment level changes.

Eight subjects (3 males and 5 females, mean age = 25.5+_5 years) voluntarily participated in this trial. Ten sites were used in each subject, these were WHO sites (16, 21, 24, 36, 41, 44) and 4 random sites.

After subject selection an impression was taken for both lower and upper jaw using rubber base material, and a model poured. The position of the selected sites were marked on the model to determine the position of the orthodontic tubes in the stent. A polyethylene plastic (Bioplast) stent of 4mm thickness was constructed on individually cast models using a vacuum forming machine. The stent was then trimmed approximately 2-3 mm coronally to the gingival margin. Two types of stent were constructed on each model, one was made according to the method used by Isidor et al.(1984) with grooves cut on the stent at the selected sites and this was called the stent without tubing. The second stent was made with orthodontic stainless steel tubing of 1.25mm internal diameter fixed into the stent as a guiding pathway for the insertion and angulation of the constant pressure probe to the selected sites, and as a fixed reference point for attachment level measurements. The same probe (constant pressure probe) was used with both stents. The measurements for both pocket depth and attachment level were repeated on 2 occasions at 2 weekly intervals. The probe was set at 0.30N probing force. Measurements were read to the nearest 0.5mm. With the stent in place, the distance was measured from the end of the tube which was the reference point to the base of the pocket and this represented the attachment level measurement with regard to the stent. The pocket depth (PD) was measured as the distance from the base of the pocket to the gingival margin, and this was taken at the same time as

attachment level measurement.

Results indicated that this modified technique using orthodontic tubing reproducibly measures pocket depth and attachment level with a compatibility between readings of 60% and 57.5% for pocket depth and attachment level respectively, while the stent without tubing showed compatibility of 43% and 50% for attachment level measurement and pocket depth respectively. Using the stent with tubing a deviation of +_1mm or less was obtained for 98% of sites and of +_2 mm for 2% of sites for attachment level measurements, as well as a deviation of 1 mm or less for 100% of sites was obtained for pocket depth measurements. Using the conventional stent without tubing, a deviation at +_1mm or less at 85% and 95% was obtained for attachment level and pocket depth measurements respectively, and a deviation at +_2mm was found in 5% of the sites for pocket depth measurement as well as a deviation equal to or more than +_ 2mm was obtained at 15% of the sites for attachment level measurements. For the stent with tubing, the mean +_SD for the differences of the replicated reading for attachment level and pocket depth was equal to - 0.075+_0.65mm, and - 0.087+_0.66mm respectively, while for the stent without tubing it was found to be 0.075+_1.07mm, and 0.25+_0.77mm for attachment level measurement and pocket depth respectively. The results indicated that splints with tubing were more accurate than the conventional onlay splints, and are suitable for longitudinal studies measuring attachment

level changes and pocket depth.

2.5.4.2 Measurement of pocket depth and attachment level changes during the longitudinal studies of untreated periodontal disease

A stent with tubing was constructed (2.5.4.1) for the upper and lower arch for each of the selected subjects. A constant-pressure probe was used to record the pocket depth and attachment level changes (2.5.4.1).

Duplicated measurements were taken for both PD and attachment level measurements at each of the selected sites for the longitudinal studies (2.2) which included the mesiobuccal site for 16, 21, 24 and the mesiolingual site at 36, 41, 44. Pocket depth measurements were taken at the same sites, as it's attachment level recordings. These duplicated measurements were repeated at each visit bimonthly during the period of the study. The mean of each pair of the duplicated measurements was used for the calculation.

In this method attachment level measurements do not rely on the location of the cemento-enamel junction (CEJ) as its identification is often complicated by its subgingival location or the presence of restorations or subgingival calculus.

2.5.4.3 Detection of attachment level changes

The change in the attachment level between each successive

visits at 2 monthly intervals, between the baseline and final visit, and over the period of observation (one year) was detected using certain methods (2.7). Significant attachment level changes were assessed retrospectively on completion of the study as described latter.

2.5.4.4 Classification of attachment level changes (attachment level grading)

A classification system for attachment level changes at the selected sites was produced. This represents the direction and the extent of attachment level change at specific sites during the longitudinal study of both periodontitis patients and the control group. Individual sites were classified according to the attachment level changes during each prescribed period into three categories:

a. Improved sites (gain in attachment level).

b. Loser sites (loss of attachment level).

c. Stable sites (no change in attachment level).

This classification was dependent on the direction and extent of the change detected according to the analytical method described below (2.7).

Figure 2.1 Modified Stent method for attachment level measurement

2.6 Collection of samples

2.6.1 Collection of the saliva

Unstimulated whole mixed saliva (at least 1 ml) was collected in a plastic 50 ml tube at each visit. The sample was then transferred to the laboratory and stored at 4 C° overnight. The clear solution carefully separated from the lower part without centrifuging and then stored at -20 C° for CRP analysis.

2.6.2 Gingival crevicular fluid (GCF) collection

Supragingival plaque was first removed from the tooth surface with a periodontal probe, during recording of the plaque index scores. This was an essential requirement prior to the collection of GCF samples in order to prevent contamination. Where the plaque was abundant (score 2 or 3) it was carefully removed using a sickle scaler with the tip kept slightly above the gingival margin in an effort to minimis the stimulation of GCF flow. GCF sampling was carried out before any other clinical recordings which could cause irritation to the tissue and serum contamination of the sample. These include pocket depth, attachment level measurements, GI, BOP and subgingival plaque sampling.

The individual site was isolated carefully with cotton wool rolls and gently air-dried in an apico-coronal direction

using a chip syringe. The area was carefully isolated and a saliva ejector was used to avoid saliva contaminating the samples. A sterile paper strip (periopaper, IDE Interstate, Amityville, NY, U.S.A) was gently inserted into the sulcus in a direction parallel to the long axis of the tooth and until mild resistance was felt, whilst care was taken to avoid mechanical trauma to the crevice. The strips were left in the crevice for one minute and then transferred to a chairside located Periotron 6000 (IDE, Amityville, NY,U.S.A) for volume determination. The sampling was repeated within three minutes after the first collection. Each periostrip was then placed in a labelled individual sterile polyethylene microcentrifuge tubes (BioRad Laboratories) into which a small, perforated inner tube had been positioned halfway down the tube. Fifty microlitres (uL) of sterile phosphate-buffered saline (0.02 M phosphate , PH 7.4) containing 0.05% Tween 20 (PBST 20) was then applied to the periostrip.

The periostrips were subsequently transported to the laboratory and stored at -40 C° prior to the elution of GCF.

2.6.3 Subgingival plaque

Bacterial samples were obtained as follows:

Supragingival plaque or debris was removed before sampling from each of the selected sites during measuring the supragingival plaque, using a clean curette followed by gingival crevicular fluid collection, then another clean

periodontal curette was introduced through the sulcus or pocket orifice as far apically as possible and the bacterial contents removed. If necessary the process was repeated several times to obtain enough material. The sample was suspended by vigorously agitating the tip of the instrument in a small screw cap vial containing 0.2-0.3 ml of sterile 0.85% sodium chloride solution containing 1% gelatin. The sampling was taken at each selected site and at each visit.

2.6.4 Detection of subgingival microorganisms using phase-contrast microscopy (PCM)

Morphological studies of the subgingival flora at each selected site for both periodontitis and control subjects were carried out using phase contrast microscopy.

The bacterial suspension was dispersed just prior to the examination by aspirating and expelling the fluid seven times through a disposable tuberculin syringe equipped with a 23 gauge needle. Special care was taken to avoid excessive air bubbles during the dispersion. In order to minimize clumping and the loss of bacterial motility, samples were prepared and the examination completed within one hour of the collection. One drop of suspension was applied to a microscopic slide and a cover slip placed over the solution. Excess fluid was removed by inverting the slide over an absorbent surface and applying moderate pressure. The slide was examined by phase-contrast microscopy at a magnification of x1200 using a 10x10 grid

eyepiece graticule during counting of the bacteria. This lens contained equal divided squares through which the bacteria could be morphologically recognized and counted. Generally from 100-200 bacteria from fields selected at random were classified into four morphological categories as follows: coccoid cells; other (curved rods with no flagella, fusiforms, straight rods, and filaments), spirochaetes (small, medium and large), and motiles. The last group included all cell types, other than spirochaetes , which exhibited motility under the microscope. This group generally comprised either straight or curved rods and on occasion fusiform and coccoid cells). Cell motility which could exhibit several characteristic patterns had to be distinguished from brownian movement or motion imparted by streaming of fluid between the slide and coverslip. All the cells exhibiting distinct flagella were also included in this group even though they were not actually moving at the time they were observed. The motiles were counted only if they were within the area which was being scanned within the field, an organism moving back and forth through the area was only counted once. Clumps of cells in which all cells could not be clearly distinguished were not included in the counts. In such cases fewer cells had to be counted than the usual minimum number of 100 cells.

The percentage of each morphotype was calculated from the absolute numbers and was used for calculation.

2.6.5 Blood sampling

Venous blood from the antecubital fossa, up to a volume of 10 mls ,was collected in glass bottles at each visit. Blood samples were transferred to the laboratory and kept at 4 C° overnight until centrifuging at 3500 rpm for 15 minutes. The serum was removed and stored at -20 C° for CRP analysis.

2.7 Data Analysis

2.7.1 Identification of attachment level change at each periodontal site

Two methods were used to identify the changes in the attachment level. These included a linear regression analysis (Goodson et al.,1982, Badersten et al.,1985d, Haffajee et al., 1983a,1986b), and a safety threshold method.

Linear regression analysis method:

By this method the slope and the intercept of a regression line were computed from a linear least squares fit of attachment level measurements as a function of time, and the slope was tested for a significant departure from zero while a threshold of projected attachment level change per unit time also had to be exceeded. Calculation of the slope of the regression line determines the rate of change over time. Determination of the probability level for the individual slope considers the variability of the individual data points about the regression line, i.e.it includes consideration of the level of reproducibility of the measurements at the specific site.

Duplicated measurements from each site were taken using the stent method described earlier (2.5.4.2) at the first visit and each bimonthly visit during the period of the study (one year). The mean of each replicate of measurements was used for data analysis. This was carried out to reduce the

probability of an error associated with these measurements. A total of 900 replicates of attachment level measurements for all the sites (150) in the control group (25 subjects) were recorded for all the visits during the study period. For the patient group (54 patients) 1920 replicated attachment level measurements were recorded for all the sites (324) for six visits during the period of study. Five sequential measurements in each group resulted from the replicated attachment level measurements between each two visits for all the sites. The sequential measurements in each group were individually subjected to regression analysis, and the extent and direction of attachment level change was determined as represented by the regression coefficient (the slope) for each site.

In order to consider that a real change occur at each individual site, the regression coefficient of the site had to departure from zero at significance level of $P<0.05$. On the basis of that a significant change in attachment level measurement per site occurred over the study period of one year, which was detected by regression analysis at probability $(P)<0.05$, three group of sites emerged. These included sites which became significantly deeper, sites which became significantly shallower, and the "not changing" sites which did not change according to trends revealed by regression analysis of probing measurements taken bimonthly on each patient (or control) for the period of the year. The criterion for "not changing" site was that a trend toward deepening or becoming shallower at $P<0.05$

significant level was not detected over the monitoring period. The number and percentages of each group of these sites is shown in Table 2.1.

Using a significance value of $P<0.05$, it was found that in the patient group, 50(15.3%) of the total sites became significantly deeper in probing attachment level, 8(2.7%) became significantly shallower, while 266(82.1%) sites showed no significant change at $P<0.05$ during the period of study (one year). In the control group, only 2(1.4%) sites become significantly deeper in their probing attachment, 3(92%) sites became significantly shallower in their probing attachment, while 145 (96.6%) sites showed no significant change over the period of study.

The standard deviations of the repeated measurements, at each visit, of all "not changing" sites were averaged and their mean was used in deciding a cut off point which was used in determining the real change for each site at each bimonthly visit and over one year. The number of "not changing" sites and the average of their standard deviations in each group was shown in table 2.2.

A cut off point which was more than three times the average of the standard deviations of the "not changing" sites was selected for both patient and control groups. For the periodontal sites in patient group, the cut off point was >1.92 mm (3x 0.64)), and later rounded up to 2mm, while for the periodontal sites in the control group this was >1.32mm (3x 0.44mm) rounded up to 1.5mm.

From the above results, a change equal to or more than 2mm

was taken as a threshold level for attachment measurement change at each periodontal site in the patient group. In order to consider that a real change of attachment level had occurred at a periodontal site, the site had to show a change which was equal to this threshold over two months between each pair of successive visits or over one year between the first and the final visit.

In the control group, a change equal to or more than 1.5mm, was taken as the threshold level for attachment measurement change at each periodontal site. In order to consider a periodontal site showed real attachment level change, it must have shown a change which was equal to this threshold over 2 months between each successive visits, or over one year between the first and sixth visit.

Another less stringent criterion was used (in our study) in detecting a real change at each periodontal site in the patient group. This included the use of a cut off point which was more than twice the averaged standard deviations of the "not changing" sites, and this was more than 1.28mm (2x0.64) rounded up to 1.5 mm.

Safety threshold method:

This is based on a reducing the false positive rate of attachment level measurements using the frequency distribution of the standard deviations of the repetitive measurements at sites which showed no significant attachment level change (the "not changing" sites) by regression analysis at $P<0.05$ (Tables 2.2, 2.3a, 2.3b).

This method was used to test the validity of the 2mm cut off point which was mentioned before.

In this method, a cut off point of 2mm for the patient group and of 1.5 mm for control group, was selected as a safety threshold in determining a real change in attachment level at each site. The 2mm safety threshold for the patient group was at least twice the standard deviations of more than 95% of the "not changing sites" determined by regression analysis. This selection was based on the chance that a false positive rate for attachment level measurements would be less than 5%, i.e. only less than 5% of attachment level changes using this threshold would be due to measurement error and this was considered the false positive rate (the number of sites which did not show a real attachment level change but they were recorded as if they did show the change). The selected safety threshold of 2mm was at least twice the standard deviations of more than 95% of the "not changing" sites and its occurrence was within less than 5% of the false positive rate.

The safety threshold for the control group was selected to be 1.5mm which was at least twice the standard deviations of more than 97% of "not changing" sites in this group, and this was also within less than 5% of the false positive rate.

This method of selecting the safety threshold was used by Lindhe et al.(1986) who used a safety threshold of more than 2mm of probing attachment level difference to identify sites with attachment loss. A cut off point of 2mm in our

study which was selected originally on the basis of averaged standard deviations of "not changing" sites determined basically by regression analysis, was confirmed when the frequency distribution of the range of standard deviations of those sites was used to determine a cut off point or safety threshold. In both methods, the safety threshold was equal to 2mm for the patient group and to 1.5mm in the control group. This difference in threshold level between patient and control groups could be due to subject variation and/or site variation (Badersten et al.1984d), as for example the mean pocket depth in the control group was significantly less than in patient group ($P< 0.05$). It has been found that probing measurements become more deep and reproducible in the deep pockets than shallow pockets (Janssen et al.1987).

2.7.2 Classification of periodontal sites according to attachment level change in the longitudinal study

A safety threshold or cut off point of 2mm and 1.5 mm was first used to classify the change in attachment level at each periodontal sites in the patient and control groups respectively, between each successive visit and between the first visit and sixth visit (over one year). On this basis, the periodontal sites were classified into three categories which are:

1.Loser sites: these were the sites that have lost

attachment level equal to or more than 2mm between each two successive visits,or over one year.

2.Stable sites: these are the sites which showed change in their attachment level at less than 2mm gain or loss between each successive visits or over one year.

3.Improved sites: these are the sites which have gained equal to or more than 2mm in their attachment level between each successive visits or over one year.

The same classification was used for the periodontal sites in the patient group when a less stringent cut off point of 1.28mm rounded up to 1.5 mm was used. The loser sites were those sites that lost equal to or more than 1.5mm attachment level, the improved sites were those which gained equal to or more than 1.5mm attachment level, while the stable sites were those which did not show a change of 1.5mm or more in their attachment level.

TABLE 2.1: The number and percentage of periodontal sites which did not change, became significantly deeper or became significantly shallower according to trends revealed by regression analysis (at $P<0.05$) of attachment level measurement taken bimonthly in all subjects within each group for the period of one year.

Subject Group	No. of Sites	Not Changing	Becoming Deeper	Becoming Shallower
Controls	150	145(96.6%)	2(1.4%)	3(2%)
Patients	324	266(82.1%)	50(15.3%)	8(2.7%)

TABLE 2.2 Means of standard deviations (SD) of repeated measurements at sites showing no significant attachment level change detected by regression analysis at $P<0.05$. The safety threshold equal to more than 3 mean of standard deviations was rounded up to the nearest mm.

Subject Group	Number of Sites	Mean SDs (mm)	Safety Threshold (mm)
Patients	266	0.64	2.00
Controls	145	0.44	1.50

TABLE 2.3a: Frequency distribution of standard deviations (SD) of the repeated probing attachment level measurements at sites showing no significant attachment level change determined by regression analysis at $P<0.05$.

Patient Group
Total sites = 266

SD Range (mm)	Number of Sites	Percentage	Accumulative Percentage
0.00-0.49	115	43.23	43.23
0.50-0.99	128	48.12	91.35
1.00-1.124	10	3.76	95.11
1.125-1.249	6	2.26	97.36
1.25-1.99	5	1.88	99.24
2.00	2	0.75	99.99

TABLE2.3b: Frequency distribution of standard deviations (SD) of the repeated probing attachment level measurements at sites showing no significant attachment level change determined by regression analysis at $P < 0.05$.

Control Group
Total Sites = 142

SD Range (mm)	Number of Sites	Percentage	Accumulative Percentage
0.00-0.49	112	77.2	77.2
0.50-0.749	23	15.8	93.0
0.75-0.99	7	4.8	97.8
1.00-1.125	3	2.2	100

2.7.3 Statistical Analysis

All statistical analyses were performed on the mainframe UNIX computing system at Edinburgh university, using the General Statistic (Genstat) package.

Various statistical analyses were used according to the requirement.

Analysis of clinical findings and microbiological data was performed using both subject and site as an experimental unit. Statistical analysis for CRP results was performed using the site as experimental unit except for saliva and serum CRP where the subject only was used for analysis.

In the case of using the subject as an experimental unit, the values of the clinical indices (PD, GCF volume and attachment level change) and microbiological data using the percentage of each bacterial morphotype (coccoid cells, other, motile rods and spirochaetes) were averaged from all the selected sites within each subject and that produced a subject mean for the variable. Data on serum C-reactive protein (CRP) and salivary CRP results were only used on a subject basis.

When the site was used as an experimental unit,the values of each of the clinical parameters, the microbiological variables (using percentages of bacterial morphotypes) and the gingival crevicular CRP results for both concentration and secretion), were averaged for all the sites in each group of subjects, and that was considered a site mean for that variable. The site mean for each subgroup of sites

(loser, stable and improved) for each variable was calculated in the similar way.

Regression analysis using Pearson correlation coefficient, Spearman rank correlation test, Chi-Square, and the analysis of variance (ANOVA) including the t-test, Fisher test (F-test) and paired-t-test, were used in current studies. These were used to seek any correlation between the changes in attachment level and each of the clinical, microbiological variable as well as CRP results. This was done using either the site or the subject (when suitable) as an experimental unit of analysis. These methods were also used to compare the differences in the clinical, microbiological and CRP results, between the classified site groups of attachment level changes (loser,stable and improved sites).

Fisher-test was used to search for any difference in the clinical, microbiological variables and GCF CRP results, among site groups at the same visit. In case the F-test showed a significance value of $F< 0.05$, the differences for any variable between the loser and stable site groups or between the loser and improved site groups of sites were tested using t-test.

Both parametric and non-parametric statistical methods (when suitable) were used to seek any possible correlation between the clinical parameters and change in attachment level for both subject and site as experimental units.

For discrete variables (plaque index, gingival index and bleeding on probing), non-parametric statistical method

were used when either subject or site was used as an experimental unit. Chi-square was used to test the correlation of these discrete variables to the attachment level change, and to compare their difference among classified groups of sites. Fisher test was used to find the correlation of these discrete variables with GCF CRP concentration and secretion.

When the site was used as an independent unit, regression analysis and Spearman rank correlation test were used to test for an association between attachment level change for each site group and the clinical, and the microbiological variables as well as GCF CRP results. The loser site group was compared with stable and improved site groups for its clinical variables, microbiological variables and GCF CRP results using a t-test.

When the subject was used as a unit of analysis, the change of attachment level between each two successive visits at 2 months intervals, and over the one year period was averaged from the selected sites within each subject, and the resultant subject mean for attachment level change was used for ranking these subjects. The values of each clinical variables (PD, GCF volume) or microbiological variable using percentage (coccoid cells, other, motile rods and spirochaetes) at the initial visit of each successive visits were averaged from the selected sites within each subject. The average was used for ranking the subject for that variable. Spearman rank correlation was used to assess the association between each of these

variables and attachment level change.

For GCF CRP results, both concentration (ng/ml) and secretion (ng/minute) were used for analysis on a site basis only, and normal logarithms for the concentration was used only when it was necessary.

For normalisation of GCF data, any negative value for the GCF volume was adjusted to 0 value for volume calculation only. Any negative value of transformed GCF volume was ignored and not used for GCF CRP level calculations for both concentration and secretion, while a 0 value of GCF volume was used for GCF CRP secretion results but not for CRP concentration results.

For GCF CRP results, when the CRP test was positive for each of the duplicated GCF samples the values were averaged, and when the test was negative for both samples the result was considered negative and was give a value of zero. In case of one GCF sample being positive and the other negative, the negative value was considered equal to 35ng/ml which was half of detectable limit (70 ng/ml) by ELISA for CRP. The value of CRP for the positive sample and the 35 were averaged to produce a mean for that sample.

GCF CRP levels were compared between loser sites and stable sites (loser versus stable sites) within each subject at initial visit and at the subsequent visit of each pair of successive visits, and also for their difference between the initial and subsequent visits. CRP levels were also compared for each site group (loser versus loser and stable versus stable), between the initial and the subsequent

visit of each pair successive visits and for the one year period. The above comparisons were carried out using ANOVA. Further more, GCF CRP levels were compared in a similar way as above using the matched pairs of loser and stable sites within each subject using paired-t-test.

A null hypothesis was suggested that there would be no association between GCF CRP levels for both secretion and concentration with attachment level change over each prescribed period. This was required to test the GCF CRP level differences between loser and stable sites recorded for pairs of successive visits or for the period of one year. The null hypothesis required that there would be no difference in GCF CRP level between loser and stable sites at the subsequent visit (where the attachment level loss could have occurred) of each paired successive visits at 2-months intervals. It also included the fact that there would be no significant difference in the CRP levels over two months (or one year) recorded for each of the loser and stable site groups.In addition, the difference in CRP levels over each paired successive visits (or over one year) should not significantly vary between the loser and stable site groups. This is the most important effect as it compares the change between the initial visit and subsequent visit at loser versus stable sites. So if these hypotheses were null particularly the last one, then CRP levels would not show any association with attachment level change, and its level would not be significantly different between the sites showing attachment level loss (loser

sites) and the non changing sites (stable sites).

The change in CRP levels, was calculated by subtracting CRP level at the subsequent visit from that at the initial visit of each pair of successive visits.

In order to investigate the possible diagnostic potential of GCF CRP levels as a test, a contingency 2x2 table (Table 2.4) was formulated based on the data of GCF CRP recorded at the subsequent visit of each pair of successive visits. The mean GCF CRP concentration (ng/ml) or secretion (ng/minute) for the loser sites at this subsequent visit was used as a threshold value for the CRP level test. In the case of using CRP concentration as a threshold value, the test was considered positive when the site demonstrated a CRP level above this designated threshold value, where if the concentration was equal to or below this threshold value the test was considered negative. The same conditions were applied when CRP secretion was used to test its use as a diagnostic aid. Specificity and sensitivity values and positive and negative predictive values were calculated using standard methods as shown in table 2.4.

Further to the above statistical methods, logistic discriminant analysis was used to find the ability of the clinical parameters, the microbiological variables, and GCF CRP levels individually or collectively to discriminate between the loser (active) and stable (inactive) site groups over each prescribed period including the one year period. This analysis was also used to find the ability of the above variables individually or collectively to predict

periodontal disease activity in the patient group over each prescribed period. In other words, to investigate their possible role, and in particular GCF CRP levels for both concentration and secretion as diagnostic predictors for the periodontal breakdown as measured by attachment level loss in our study.

Throughout this thesis, the term 'paired successive visits' is used to describe any pair of the consecutive visits which took place at intervals of two months during the course of the study. For example Visit 1 and Visit 2 (two months apart) are considered 'paired successive visits', where Visit 1 is the initial visit of the pair and Visit 2 is the subsequent visit.

TABLE 2.4 2x 2 table for CRP diagnostic test and its characteristics.

	Disease Present	Disease Absent
Positive CRP test	True positive (a)	False positive (b)
Negative CRP test	False negative (c)	True negative (d)

Positive predictive value = a/ (a + b)

Negative predictive value = d/ (c + d)

Sensitivity = a/(a + c)

Specificity = d/(b + d)

CHAPTER THREE

Laboratory Materials and Methods

3.1 Materials

All chemicals were analytical grade and obtained from BDH chemicals Ltd., Poole, Dorset, England, unless otherwise stated.

3.1.1 Buffers

3.1.1.1 Coating Buffer

Carbonate-bicarbonate buffer (pH 9.6): this was prepared by dissolving 1.59g Na_2CO3, 2.93g $NaHCO_3$, and 0.2g of NaN_3, made up to 1 litre with distilled water, and the pH was adjusted to 9.6. This buffer was then stored at 4 C° and used for two weeks only .

3.1.1.2 Phosphate buffered saline (PBS)

This was prepared by dissolving 8.0g of NaCl, 0.2g of KH_2PO_4, 2.9g of $Na_2HPO_4.12H_2O$, 0.2g of KCl made up in 1 litre of distilled water, and the pH was adjusted to 7.4.

3.1.1.3 Washing and diluting Buffer (PBST-20)

This is a phosphate buffered saline plus 0.05% w/v tween, prepared by dissolving 8.0g of NaCl, 0.2g of KH_2PO_4, 2.9 of $Na_2HPO_4.12H_2O$, 0.2g of KCl,0.5ml of tween 20 (Sigma Chemical Company Ltd, Poole, Dorset),made up to 1 litre of distilled water. The pH was adjusted to 7.4.

3.1.1.4 Phosphate citric buffer

This was prepared by adding 25.7mls of 0.2 M dibasic sodium phosphate, and 24.3mls of 0.1 M citric acid to 50 ml of deionized water made up to 100 mls. The pH was adjusted to 5.0.

3.1.1.5 Fetal Bovine serum

This was bought from Boehring (Boehringer Mannheim, Diagnostics and Biochemical Co.Ltd, Lewes, East Sussex, U.K).

3.1.2 Purified standard C-reactive protein and antisera for enzyme-linked immunosorbant assay (ELISA)

3.1.2.1 Rabbit Anti-Human C-Reacive Protein (IgG Fraction of Antiserum Developed in Rabbit

This was bought from Sigma (Sigma Chemical Company Ltd, Poole, Dorset, UK).

3.1.2.2 Goat Anti-Rabbit IgG (Whole Molecule) Horse Raddish Peroxidase Conjugate (affinity isolated antigen specific antibody developed in goat)

This was bought from Sigma (Sigma Chemical Company Ltd, Poole Dorset, UK). It had an enzymatic activity of 35 purpurogallin units/ml.

3.1.2.3 Goat Anti-Human C-Reactive Protein developed in goat

This had a concentration of 6.3mg/ml and was bought from Sigma (Sigma Chemical Company Ltd, Poole, Dorset, UK).

3.1.2.4 Substrate

Each tablet contained 4mg of o-Phenylenediamine dihydrochloride (OPD). This was bought from Sigma (Sigma Chemical Company Ltd, Poole, Dorset, UK).

3.1.2.5 Substrate buffer solution

This was made by dissolving 1 tablet of the substrate (OPD) in each 20 mls of phosphate citric buffer at pH 5.0. Four microlitres of 100% volumes hydrogen peroxide were immediately added to each 20 mls of phosphate citric buffer prior to addition of the substrate.

3.1.2.6 Standard C-Reactive Protein (STD CRP)

This was bought Boehring (Boehringer Mannheim, Diagnostics and Biochemical Co.Ltd, Lewes, East Sussex, U.K). It a had concentration of 79 mg/L.

All the standards and antisera for ELISA assays came in liquid form except Rabbit anti-human CRP which came in the

form of lyophilised powder and was reconstituted according to the manufacurer's instructions. All reagents, after reconstitution if required,were aliquoted and stored at -20 C° until used. Reagents were replaced regularly to avoid expiration.

3.1.3 Microtitration plates

These were microtitre plates bought from Dynatech Laboratories Ltd.Sussex, UK.

3.1.4. Multiskan Reader II

This was purchased from Flow Laboratories, Rickmansworth U.K.

3.2 Methods

3.2.1 Calibration of Periotron 6000

The Periotron was calibrated on a bimonthly basis by duplicate measurements of volumes using normal blood serum from 0.05 to 1.0uL on periopaper strips (periopaper, IDE Interstate, Amityville, NJ,U.S.A.). At the time of each patient sampling additional duplicate determinations of 0.3 and 0.5uL were also made.

The reciprocal jaws were cleaned with 95% methanol and allowed to air dry before the filter paper strip was inserted between its jaws. Prior to application of a sample a dry clean periopaper was placed between the electrodes of the Periotron was then adjusted to zero. The paper was then removed and a measured volume of serum was placed on the end of the strip using a one microlitre Hamilton syringe (Hamliton Bonaduz, AG, CH-7402, Switzerland). This syringe was used to deliver to the filter-paper strips volumes ranging from 0.05 to 1.35uL at 0.05 increments. The paper was replaced in the Periotron 6000 (IDE, Amityville, New York, U.S.A.) and the scale value read after 16 seconds. This was repeated ten times for each volume. The Periotron readings for all volumes were expressed as the mean +_ standard deviation of all ten measurements. Linear regression analysis was used to generate lines representing the relationship of volumes to readings on the Periotron 6000 (Figure 3.1).

Using linear regression analysis, the points plotted for serum were resolved into two lines. The first line being determined by fluid volumes from 0.05 to 0.4uL, and the second line by fluid volumes from 0.4 to 1.35uL. The slope, intercept, and the coefficient of correlation for each line in both scales were provided in Figure 3.1. The slope of the lines determined by volumes below 0.4 was significantly ($P< 0.01$) less than the slope of the line determined by volumes greater than 0.4. An important consideration is the volume of fluid corresponding to each unit reading on the periotron 6000. Using the slope of the line determined by smaller volumes of fluid (0.05- 0.4uL), each periotron unit was equal to 0.00484 uL for serum ($r= 0.9979$) while for the line determined by volumes above 0.4uL, the slope was corresponded to 0.00923 ($r= 0.9962$) uL per unit of reading. The study showed a reasonably accurate assessment of GCF volume from 0.05 to 0.4uL as each unit reading corresponded to approximately 0.005uL, was consistent with that of the manufacture, but not for volumes above 0.4. Our results for those volumes (0.05-0.4uL) are also consistent with those by Lamster (1985b) and Hinrich et al.(1984).

3.2.2 Determination of GCF volumes

Conversion of a specific number of units on the Periotron to an actual GCF volume requires consideration of both the slope and the intercept.

A calibrated curve was constructed for the Periotron 6000 to determine the actual volume of GCF on each filter strip (Fig.3.1). The graph is based on the relationship of unit readings on the Periotron to known volumes of serum delivered onto the strip. This calibration graph indicated that each periotron unit from 0 to less than 75 units corresponded to 0.00484uL, and each periotron unit above 75 was corresponded to 0.00923uL.

The conversion of specific unit readings on the periotron 6000 to the actual volume of GCF sampled on a filter strip was made by using the equation for that section of calibrated graph. The following equation was used for the calculation:

Y = AB + C. where:

Y = The actual volume sampled on periostrip to be determined.

B = the slope of the line relating fluid measurements to fluid volumes.

A = the Periotron reading.

C = the measurement intercept when the Periotron reading is 0.

When calculating the actual volumes of GCF, the slope, and the intercept of the calibration graph were used for each cut off volume.

Fig.3.1 showed the relationship of unit readings on the Periotron 6000 to actual volume of fluid which was drawn by the graph. Linear regression analysis was used to fit lines to the data. The data were resolved into two lines

(0.05 to approximately 0.4uL, 0.4 to 1.35uL), and the slope , intercept and correlation coefficient (r) were provided. The boundaries of these lines as based on the abscissa (unit readings on the Periotron) for serum were 0-75, and 75-175. Based on these cut off points related to the boundaries of these two lines, the following mathematical equations which are consistent with above equation, were used to convert the periotron reading in the actual volumes which used calculations:

I.If the reading was equal or below 75, the following equation was used:

$Y = (A \times 0.00484) + (-0.005)$.

II. if the reading was above 75, the following equation was used:

$Y = (A \times 0.00923) + (-0.32)$.

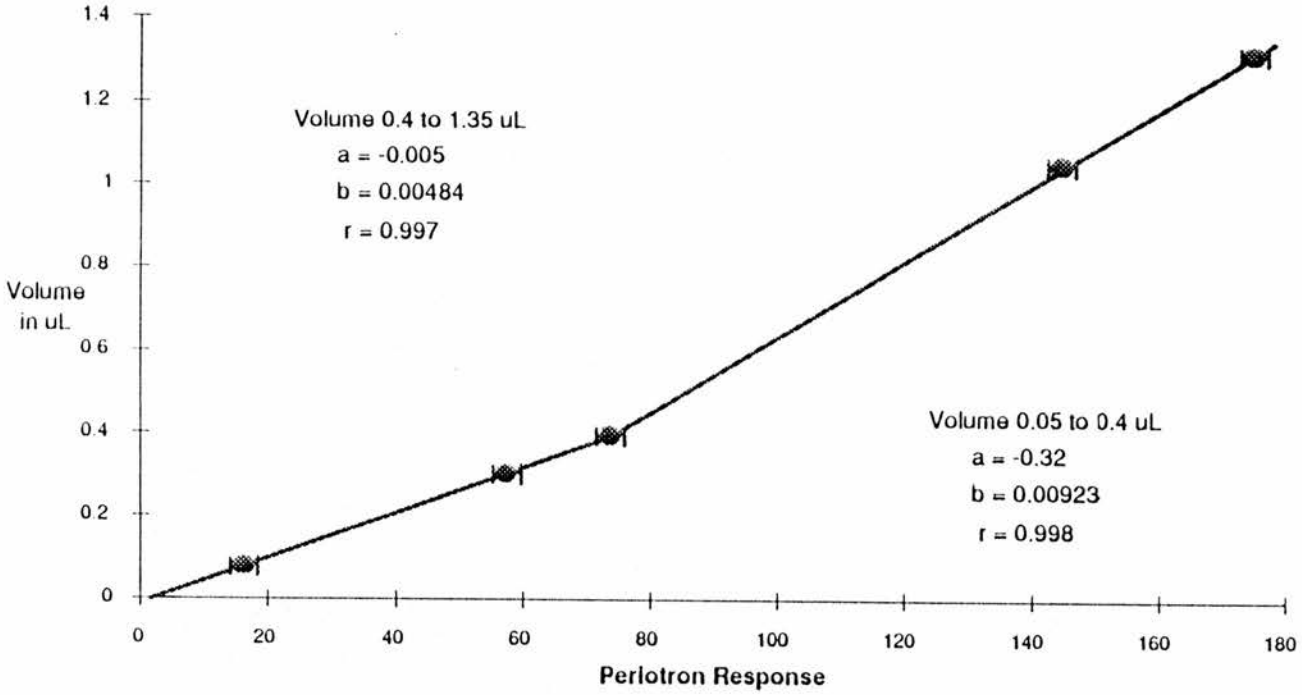


Figure 3.1 Calibration Curve for the Periotron 6000 at the volume range 0.05 to 1.35 uL.
Each point represents the mean of ten measurements (+- SD)
Linear Regression analysis was used to plot the line of best fit and regression coefficients

a = intercept
b = volume co-efficient
r = Pearson correlation co-efficient
SD = Standard Deviation

3.2.3 Elution of GCF from periopaper

A method for elution of static GCF was developed and was shown to provide nearly total recovery of CRP activity from the filter papers. The GCF samples after collection (2.6.2) were recovered from the strips via a centrifugal elution technique. The 50 uL of PBST used for storage of periostrip was removed from the inner tube inside the microcentrifuge tube and relayered on the filter strip. The tubes were centrifuged at 13,000xg for 30 seconds, followed by three additional extractions with 50 uL of PBST. The strip, which was still retained in the perforated inner tube, was then discarded and the eluted 200 microlitre, in the lower part of the tube, transferred to a fresh vial and stored frozen at -40 C° until required for subsequent analysis of CRP which was normally performed within two months of collection. The volume on the strips was estimated by means of Periotron 6000 units and reference to the calibration curve.

3.3 Development of a modified ELISA technique for quantification of CRP in Serum, Saliva and Gingival Crevicular Fluid

A double sandwich solid phase enzyme-linked immunosorbent assay (ELISA) was developed to detect and measure CRP in GCF,saliva and serum. This is a modified technique of the ELISA used by Salonen (1982) for measuring serum CRP, with an improvement to increase its sensitivity limits and this could be used for measuring CRP in small volumes such as 0.1-0.2uL of GCF samples.

3.3.1 Sandwich ELISA methodology

Method:

First step:

This included coating the microtitre plate with the first antibody, the goat antihuman CRP antiserum which is specific to the antigen (CRP).

Microtitre plate (Dynatech Laboratories, Sussex, U.K) was coated with 50 microlitre/well of goat antihuman CRP antiserum at 1/5000 in coating buffer (carbonate bicarbonate buffer) at pH 9.6. The plate then incubated overnight at 4 C°.

Second step:

This included the addition of the test sample (serum, GCF, saliva), and purified standard serum CRP to the plate where any antigen (CRP) present in the sample was captured by the immoblised antibody. In this step, the plate was washed with washing buffer (PBST 20) three times with 30 seconds

intervals, and dried in the air. 50uL of diluted antigen (standard serum CRP) or diluted test sample in PBST 20 were added per well and incubated for 90 minutes at 37 C°.

The test sample for GCF, serum, and saliva were diluted at 1/200, 1/2000, and 1/2 respectively. The first vertical column of the plate was used for 8 controls which were antigen-free. The vertical second and final columns were used for serial two-fold dilutions of the purified standard CRP, resulting in a total of 8 standard solutions within the working range for the CRP assay from 8.976 to 0.070 ng/ml. This allowed the construction of a standard curve from which sample antigen quantities could be found. The standards were run in duplicate,and a total of 24 samples of antigen were run in triplicate horizontally in each plate.

Third step:

This included incubation with the second specific antiserum, the rabbit anti-human CRP antiserum. In this step, the plate was washed three times with PBST 20, and dried. 50uL of rabbit anti-human CRP at dilution 1/20000 in PBST 20, was added for each well, incubated for 90 minutes at 37 C°.

Fourth step:

This included the incubation with horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG. In this step, the plate was washed three times by PBST 20, dried in the air, and 50uL of horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG at dilution of 1/500 in PBST 20 was added

to each well. The plate was incubated for 120 minutes at 37 C°.

Fifth step:

This included addition of the substrate (OPD) to the plate, stopping the reaction and the plate reading. The plate was washed three times with PBST 20, dried in the air, and 100uL of the substrate (OPD) solution was added to each well. The reaction was stopped within 25 minutes using 50 uL/well of 0.2M H_2SO_4. The plate was then read at 492nm blanked on the first column (the controls), and the optic densities obtained using a Multiscan II reader which connected directly to the an IBM PC computer with a computing ELISA programme to give absolute reading of CRP as ng/ml.

In all steps the incubating buffer (PBST 20) contained fetal bovine serum (FBS) at 5% w/v and 0.05% w/v of Tween 20, except the substrate solution and the coating antibody step.

3.3.2 Establishment of CRP sandwich ELISA

A series of experiments were conducted in order to establish conditions for the ELISA. The standard chess (check)-board type dilutions were used for defining the optimal condition for goat, rabbit and HRP conjugated antiserum dilutions.

Serial Chess-board type dilutions of goat antihuman CRP antibody (1/100, 1/1000, 1/3000, 1/5000, 1/10000) in

coating buffer (carbonate bicarbonate buffer), Rabbit antihuman CRP antibody (1/5000, 1/10000, 1/20000, 1/40000), and HRP conjugate (goat anti rabbit IgG conjugate) at 1/100, 1/250, 1/500, 1/1000) in PBST 20 incubating buffer were used. The optimal conditions for each of these reagents were obtained where the specific antisera were not in excess of the antigen (CRP), where there was a minimum background (non-specific binding). These included 1/5000 dilution in coating buffer for goat anti-human CRP antiserum, 1/20000 dilution in PBST 20 for rabbit anti-human CRP antiserum, and 1/500 dilution in PBST 20 for HRP conjugate goat anti-rabbit IgG antiserum , and all (except goat) antiserum gave best results with incubation temperature at 37 C°. Incubation with goat anti-CRP antibody overnight at 4 C°, was used because this gave more uniform results. Incubation for 90 and 120 minutes with the standard CRP or specific rabbit antiserum gave a similar standard curve resolution. 120 minutes for incubation with HRP conjugate gave better standard curve resolutions and better results for the test samples than 90 minutes particularly when the conjugate at 1/500 in diluting buffer was used. These conditions gave low non-specific binding (optic density < 0.01) in the controls and maximum optic density (OD) in the lowest standards 25 minutes after adding the substrate. So incubation for 90 minutes with standard/ specific antisera, and incubation for 120 minutes with HRP conjugated serum was selected for the ELISA.

The best results were based on the best curve resolutions

and the very low non-specific binding in the controls. Also the optimal conditions included maximum OD in the presence of the antigen (Standard CRP).

Various percentages of the blocking agent, the fetal bovine serum (FBS) at 10% w/v, 5% w/v and 1% w/v were tested in their ability to block the non-specific binding and reduce the background. The 5% FBS was found to give the optimal results for ELISA with minimal background and high sensitivity. Blocking with FBS at 5% w/v in all reagents, with exception of coating antibody and substrate solution, during incubation was judged to be sufficient.

The detectability limit of the assay, was defined as the lowest concentration of the standard antigen (CRP) which gave optic densities significantly different from that of the controls. In order to make the assay more accurate and precise, the best concentration of standard CRP was selected, on the basis that the lowest standards or those values of the linear portion of the standard curve gave an optic density significantly higher than that of the controls.

Various working ranges of purified CRP (standard CRP) were tested to construct the reference curve. These included 18.0-0.14ng/ml, 9.00-0.077ng/ml, 8.977-0.070 ng/ml and 4.488-0.350ng/ml) of purified STD CRP. The last working range resulted in less significant differences at the lowest portion of the curve from that of controls when compared to the standard CRP concentration range of 8.977 to 0.07 ng/ml and thus the second one was selected for the

construction of the curve. This range gave best resolution for the standard curve, and significantly higher optic densities for the lowest standards than those of the controls. The working range of the standard antigen was produced by serial two fold dilutions of the standard antigen in PBST 20, resulting in 8 standard dilutions for the construction of a standard curve (Figure 3.2). The resolution of the curve drawn from standard CRP concentrations plotted against their ODs were improved when the working range of 4.488-0.035ng/ml was increased to the range of 8.977-0.070ng/ml. This was carried out to make the difference between the OD of the lowest standards of the linear portion (precision part) of the standard CRP curve at the second working range different significantly from that of the controls, in comparison with the first working range. This working range had a significantly different optic density for the last two values when compared with ODs of the controls which have very low non-specific binding (background) < 0.1 with H_2SO_4 and < 0.01 before stopping the reaction 25 minutes after the addition of substrate.

Figure 3.2 showed the typical calibration curve for standard CRP at concentrations ranging from 8.977-0.070ng/ml, plotted against their corresponding optic densities. The least squares method was used to plot the line of best fit and the correlation coefficient ($r > 0.99$ was obtained for the assay. Linear function of the concentration $C = A + B \times OD$, where OD= optic density, C=

the concentration, A= the intercept and B= the slope (the best fitted line of concentration against the OD) was used for the determination of sample antigen quantities. A similar standard curve was generated automatically by the computerised ELISA programme, and a new line fitted each time an assay was run. The sample antigen was quantified directly from this calibrated curve through the program.

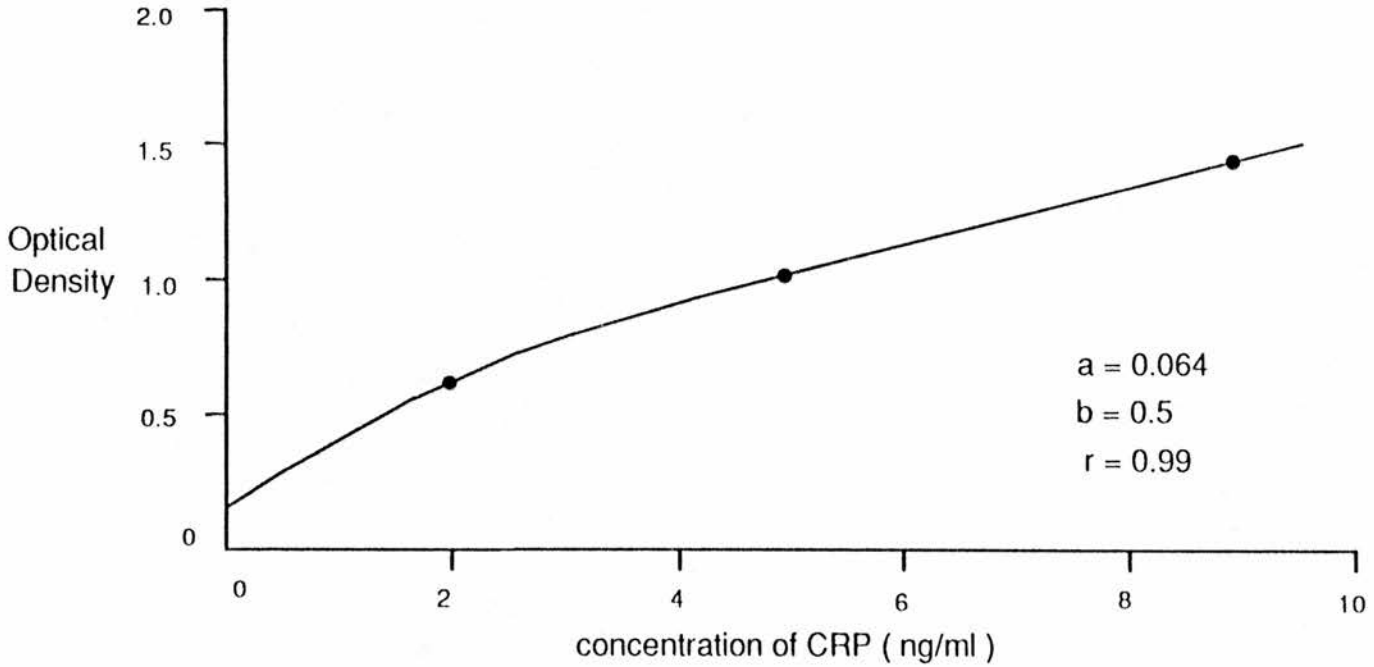


Figure 3.2 Typical Calibration curve for Standard CRP Sandwich ELISA
(working range 0.07 - 8.977 ng/ml)
Optical Densities were plotted against logn concentrations.
The regression co-efficients are shown.

a = intercept
b = concentration co-efficient
r = co-efficient of correlation

3.3.3 Pilot study in the quantification of C-reactive protein (CRP) in serum, saliva and GCF using double sandwich ELISA

3.3.3.1 Preliminary experiments to detect and quantify CRP in GCF

Studies were conducted to find if CRP was present in GCF or not. Small volumes of GCF were collected from healthy sites (less than 0.1uL for most of them), and the minimum amount of fluid required to run ELISA assays for antigen determination in triplicate is 150uL (50uL per well). Thus eluting sampled GCF in 1/200 of PBST 20 was tried first. In order to test for the presence of CRP in GCF and to establish an elution volume and working dilution of the sampled GCF for quantification of CRP by ELISA, 100 samples of GCF were tested with various volumes (Table 3.1) randomly collected from periodontal sites from both healthy controls and patients with periodontal disease. These samples were tested for the presence of CRP, and the elution/dilution procedure for GCF was established. All the samples were eluted first in 200 uL of PBST 20 as described earlier (3.2.3), and further diluted to 1/600 in PBST 20. The GCF samples were tested for CRP using ELISA for both at 1/200 and 1/600 dilutions in PBST 20.

The results (Table 3.1) showed that CRP is present in GCF and this is supported by the finding of Sibbera et al. (1991) who detected CRP by an immunodot method in GCF from both healthy sites and from sites with periodontal disease.

The results also showed that an increase in the elution factor from 1/200 to dilution of 1/600, will result in decreasing the incidence of CRP detection in GCF samples from 37% to 20%. It was also found that the range and the mean of the volumes which gave a positive test for CRP were increased from the range of 0.12-1.0 uL and mean of 0.317 uL for the positive samples at elution of 1/200 to the range of 0.305-1.0 uL and mean of 0.40uL for the positive samples at dilution of 1/600. CRP at elution of 1/200 could not be detected in volumes below 0.121 uL, with a mean of 0.317 uL, while at dilution 1/600 CRP could not be detected at volumes up to 0.305uL and at mean volumes of 0.40 uL.

TABLE 3.1. Gingival crevicular fluid CRP levels (ng/ml) as determined by ELISA in the original GCF eluate at 1/200 in PBST 20 and 1/600 in PBST 20. Incidence of CRP positive test is shown. Negative results indicate that CRP levels cannot be detected by ELISA.

	GCF elution at 1/200 in PBST 20 (total sample = 100) (range of volume = 0.02-1.0 uL)		GCF dilution at 1/600 in PBST 20 (total samples = 100) (range of volume = 0.02-1.0 uL)	
CRP test	Positive	Negative	Positive	Negative
Number of samples	37	63	20	80
GCF volume range (uL)	0.121-1.00	0.020-0.350	0.305-1.00	0.020-0.52
GCF volume mean (uL)	0.317	0.115	0.40	0.330
CRP (concentration (ng/ml)	95.3-560.6	-	37.6-210.00	-

In order to confirm the presence of CRP in GCF and to find if the elution of 200uL is better than 250uL, another 100 samples of GCF were eluted (3.2.3) in 250uL and were also further diluted to 1/500 in PBST 20. These samples were tested for the presence of CRP by ELISA. These samples had various volumes (Table 3.2) and had been randomly collected from various sites from both healthy subjects and periodontally diseased patients. The results confirmed the presence of CRP in GCF, and indicated that increasing the dilution of GCF resulted in decreasing of the incidence of CRP in GCF from 20% at elution 1/250 to 11% at dilution of 1/500. A positive CRP test was only found at volumes equal to or more than 0.213 uL with elution of 1/250, and at volumes equal to or more than 0.37uL when GCF were diluted up to 1/500.

The results from both experiments using elution at 1/200, 1/250 and dilution at 1/600, 1/500 in PBST 20, confirmed CRP presence in GCF, and that the developed ELISA could be used to detect its presence. These data also indicated that elution of GCF samples in 200 uL was most suitable for detection of GCF CRP, if present, in all the volumes as both the lowest and highest concentrations of samples tested were found to be within the working range of the standard CRP. On the other hand, it seems that 1/200 elution would avoid further dilution of volumes particularly at 0.1-0.2uL and would avoid the chance of missing the CRP, if present. It would however, help to detect the CRP, if being present in small volumes

particularly at healthy sites or those with early inflammation. This was later proved when no volume below 0.1uL showed the presence of CRP, and no volume even more than 1uL showed CRP concentration outside the working range of the standard CRP using elution of GCF at 1/200 uL with PBST 20.

The volume (elution) to 1/200 from the technical point of view is the lowest volume that is required for the assay (ELISA).

The experiments also indicated that the developed ELISA can only detect CRP, if present, in any volume equal to or more than 0.1uL. Any volume below that showed no CRP present, and this either was due to the dilution factor and limited sensitivity of ELISA or more likely the concentration was too small to be detected or there was no CRP.

TABLE 3.2. Gingival crevicular fluid CRP levels (ng/ml) as determined by ELISA in the original GCF eluate of 1/250 in PBST 20, and at dilution of 1/500 in PBST 20. Incidence of CRP positive tests was shown. Negative results indicate that CRP levels at that volume cannot be detected by ELISA.

	GCF elution at 1/250 in PBST 20 (total samples = 100) (volume range = 0.035-1.05 uL)		GCF dilution at 1/500 in PBST 20 (total samples = 100) (volume range = 0.035-1.05 uL)	
CRP test	Positive	Negative	Positive	Negative
Number	20	80	11	89
GCF volume range (uL)	0.213-1.05	0.035-0.420	0.370-1.05	0.035-0.530
GCF volume mean (uL)	0.340	0.220	0.430	0.346
CRP concentration (ng/ml)	88.0-610.0	-	49.0-325.0	-

In order to find if CRP in GCF responds in the same way as the standard CRP, five samples (sample 1 to 5) of GCF were collected and eluted (3.2.3) originally in 200uL of PBST 20, and were further diluted in 1/600,1/1800 of PBST 20. Another six samples (sample 6 to 11) were collected and eluted (3.2.3) originally in 1/250uL of PBST 20, and were further diluted at 1/500, 1/1000 in PBST 20.
As shown in Table 3.3 acceptable dilution profiles were obtained for CRP in all the samples. This indicated that CRP in GCF responds in a similar way to the highly purified standard CRP and all the concentrations were within the working range when elution 1/200 was used. This also indicated that the developed assay (ELISA) could be used for GCF CRP quantification.

3.3.3.2 Assessment of the recovery of CRP protein from periopaper <u>in vitro</u>

In order to assess the suitability of periopaper as a means of collecting GCF for subsequent CRP analysis, the recovery of CRP applied to this strip was investigated. Serum samples containing known concentrations of CRP were used for this investigation. Using a one uL Hamilton syringe, one uL of the serum was applied to each periopaper (10 strips). Each strip was then transferred to a plastic micro-centrifuge tube and the collected fluid was eluted into 200uL of PBST 20 (3.2.3). The eluted samples were then further diluted to 1/2000 in PBST 20 in order to achieve

TABLE 3.3. Gingival crevicular fluid CRP levels (ng/ml) as determined by ELISA in serial dilutions of the original GCF eluates at 1/200 and 1/250 uL in PBST 20. Results 'below' indicate that the CRP level cannot be detected by ELISA at that dilution.

Sample	GCF Volume (uL)	Dilution Factor	CRP Concentration (ng/ml)
1	0.787	1/200	238
		1/600	90.3
		1/1800	49.6
2	0.208	1/200	277.5
		1/600	98.3
		1/1800	below
3	0.365	1/200	713
		1/600	241.2
		1/1800	80.23
4	0.529	1/200	333.78
		1/600	132.4
		1/1800	below
5	0.252	1/200	55.55
		1/600	below
		1/1800	below
6	0.126	1/250	115.8
		1/500	below
		1/1000	below
7	0.279	1/250	66.7
		1/500	below
		1/1000	below
8	0.213	1/250	274
		1/500	147.6
		1/1000	below
9	0.373	1/250	554.8
		1/500	286.2
		1/1000	94
10	0.252	1/250	141
		1/500	82.2
		1/1000	below
11	0.210	1/250	49.3
		1/500	below
		1/1000	below

concentrations in the eluate within the working ranges of the sandwich ELISA. The concentrations of CRP in the serum in the original 200uL eluate for each of the strips was compared against that in the 200uL (10 samples) of PBST 20 directly delivered to the plate after dilution in 1/2000 uL of PBST, and the percentage of the efficiency of recovered CRP was calculated. The results showed that 96.2% of CRP in the eluted serum applied to periostrips was recovered.The mean +_SD of CRP concentration for eluted serum was 19.3+_0.8 ug/ml, and for non eluted serum sample was 20.1+_0.7 ug/ ml. The results indicated that this method of elution and recovery was sufficient to recover almost all of the CRP from periopaper, and that the periostrip is suitable for collecting GCF for CRP analysis.

3.3.3.3 Quantification of serum CRP

In order to find if the developed ELISA could be used for determination of serum CRP, the following experiments were conducted.

In the first experiment, 50 samples of serum were used to find the working dilution of serum for CRP determination. The samples were diluted at 1/1000, 1/2000, and 1/4000 in PBST 20, and tested for CRP by ELISA. The results (Table 3.4) indicated that dilution at 1/2000 is the best one for the determination of CRP in serum as it is the lowest dilution that gives a high incidence of positive tests within the working range of standard CRP. During subsequent experiments when the test serum CRP was detected above the working range, the next serial two-fold dilution of the sample was rerun to detect the CRP.

In the second experiment, five samples of serum were collected and tested for CRP by ELISA. Serial dilutions of these sera were tested at 1/2000, 1/4000, 1/8000, and 1/16000 in PBST 20 (Table 3.5).

It was found that the concentrations of CRP in serum responded in a similar way to CRP standards in ELISA. This indicated that the developed ELISA was suitable for measuring serum CRP.

3.3.3.4 Comparison of ELISA with Laser nephelometry in CRP determination

The developed ELISA was compared with Laser nephelometry (LN) for the detection of CRP.

Thirty sera from cystic fibrosis patients were kindly granted to me from Edinburgh Royal Infirmary. These contained CRP at concentrations of 10.5-45.5 mg/L with a mean of 28.5mg/L. Their concentrations were already determined by the LN technique. All 30 sera were diluted at 1/8000 in PBST 20, and subjected to CRP determination using ELISA. It was found (Table 3.6) that their concentrations ranged from 8.5 to 41.2mg/L and had a mean of 26.3mg/L. Using percentage efficiency calculations, it was found that ELISA can quantify up to 92.2% of the total amount of CRP detected by LN. The correlation between both methods in quantifying CRP was high (r= 0.93). It could be concluded that the developed ELISA was valid and suitable for determination of CRP, even at high concentrations.

TABLE 3.4. CRP levels as determined by ELISA in sera at serial dilutions in PBST 20. The result 'below' or 'above' indicates that CRP levels cannot be detected at that dilution as it was outside the working range of standard CRP. Incidence of positive CRP test is shown.

	Dilution Factor					
	1/1000		1/2000		1/4000	
CRP test	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Number	45	5	50	-	39	11
CRP concentration (mg/L)	0.28-8.50	above	0.15-5.30	-	0.12-2.10	below

Pos. = Positive
Neg. = Negative

TABLE 3.5 CRP concentrations (mg/L) in sera as determined by ELISA at two-fold serial dilutions (1/2000-1/16000) in PBST 20. Results 'below' or 'above' indicate that CRP levels cannot be detected by ELISA as they were outside working range of standard CRP.

Sample	Dilution Factor	Serum CRP concentration (mg/L)
1	1/2000	11.122
	1/4000	5.661
	1/8000	2.790
	1/16000	1.410
2	1/2000	9.144
	1/4000	4.680
	1/8000	2.395
	1/16000	1.205
3	1/2000	0.258
	1/4000	0.146
	1/8000	below
	1/16000	below
4	1/2000	above
	1/4000	8.65
	1/8000	4.390
	1/16000	2.200
5	1/2000	0.144
	1/4000	below
	1/8000	below
	1/16000	below

TABLE 3.6. Serum CRP concentrations as determined by both ELISA and Laser Nephelometry (LN) method. A dilution of 1/8000 in PBST 20 for each sample was used for ELISA. Percentage of efficiency of ELISA in measuring CRP was 92.2%.

	Method of CRP Detection	
CRP Levels	ELISA (number = 30)	Laser Nephelometry (number = 30)
Mean CRP concentration (mg/L)	26.301	28.50
Range of CRP concentration (mg/L)	8.50-41.20	10.5-45.50

3.3.3.5 Quantification of CRP in saliva

Saliva samples were used to test for the presence of CRP. Fifty samples of unstimulated whole saliva were randomly collected and used for CRP analysis by ELISA. Up to 2mls were collected for each sample from controls and patients with periodontal disease. Samples were left overnight at 4 C°, and then the clear solution removed and transferred to another clean tube for CRP analysis. The samples were then diluted in serial dilutions at 1/2, 1/4, and 1/8 in PBST 20, and tested for CRP using ELISA.

The results (Table 3.7) indicated that CRP was present in the saliva. The data also showed that a dilution at 1/2 was found to give the best results, and showed a high incidence of positive CRP tests which was 53%(28) of the total samples. The incidence of positive CRP was found to be decreased with increasing dilution factors (Table 3.7).

In order to find if the CRP in saliva responded in a similar way to that for standard CRP using ELISA, five positive samples were selected and retested for CRP at dilutions of 1/2, 1/4, 1/8 in PBST 20. It was found (Table 3.8) that salivary CRP responded in a similar way as standard CRP, within the working range, and as the dilution factor increased the concentration decreased. These results indicate that the developed ELISA is suitable for detecting of CRP in the saliva.

TABLE 3.7 CRP concentration (ng/ml) in saliva samples as determined by ELISA at double serial dilutions (1/2, 1/4, 1/8) in PBST 20. The 'negative' result indicates that CRP level cannot be detected at that dilution as it was below the working range of standard CRP.

	Dilution Factor					
	1/2		1/4		1/8	
CRP test	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Number	28	22	18	32	8	42
Mean CRP concentration (ng/ml)	0.382	-	0.290	-	0.221	-
Range of CRP concentration (ng/ml)	0.143-4.50	-	0.173-2.00	-	0.150-1.150	-

Pos. = Positive
Neg. = Negative

TABLE 3.8. CRP levels (ng/ml) in saliva samples as determined by ELISA at serial dilutions (1/2, 1/4, 1/8) in PBST 20. The result 'below' indicates that the CRP level cannot be detected at that dilution as it was below the working range of standard CRP.

Sample	Dilution Factor	CRP concentration (ng/ml)
1	1/2	0.370
	1/4	0.196
	1/8	0.132
2	1/2	1.166
	1/4	0.601
	1/8	0.317
3	1/2	1.850
	1/4	0.996
	1/8	0.512
4	1/2	0.142
	1/4	below
	1/8	below
5	1/2	0.152
	1/4	below
	1/8	below

CHAPTER FOUR

RESULTS

4.1 Attachment level changes in both periodontal patient and healthy control groups during the longitudinal study

4.1.1 Incidence of attachment level change over one year determined by linear regression analysis method

Fifty four periodontal patients on maintenance therapy at 1-3 months recall and 25 healthy control dental students with no evidence of periodontal diseases were involved in present study. Six sites within each subject was selected (2.2) and these resulted in 324 sites for the patient group and 150 sites for the control group which were studied over one year.

Attachment level measurements were taken in duplicate at each of these sites, at each bimonthly visit, and at the baseline visit (first visit) using the stent method which was described earlier (2.5.4.2). A total of 1920 and 900 replicated measurements were recorded for all the sites at all visits in patient and control group respectively. These replicated measurements at each visit for each site were averaged, and the site mean was used for data analysis. The replicated measurements resulted in five paired sequential measurements for each group during the whole year.

The five sequential attachment level measurements were subjected to linear regression analysis (2.7.1) and the direction and extent of attachment level change over the study period (one year) was determined. The projected change which was the rate of change over one year was

determined by the regression coefficient (the slope) for each site, and this coefficient departed from zero at a significance value of $P<0.05$.

On the basis of a significant change in attachment level measurement per site occurring over the study period of one year, which was detected by regression analysis at probability ($P< 0.05$), three group of sites emerged. These included sites which became significantly deeper, sites which became significantly shallower,and the "not changing" sites which did not change according to trends revealed by regression analysis of probing measurements taken bimonthly for each patient (or control) for the period of the year. The criterion for a "not changing" site was that a trend toward deepening or becoming shallower at $P<0.05$ significance level was not detected over the monitoring period. The results of regression analysis for attachment level changes are shown in Table 4.1.

It was found that in the patient group 50(15.3%) of the total sites in 25 subjects became significantly deeper (mean change was -0.42 mm) in probing attachment level, 8 (2.7%) of sites in 7 subjects became significantly shallower (mean change was +0.39 mm), while 266(82.1%) of the sites showed no significant change at $P<0.05$ during the period of one year study. In the control group, only two subjects showed attachment level loss over one year and 2(1.4%) of total sites become significantly deeper (mean change was -0.2mm) in their probing attachment level, 3(2%) sites became significantly shallower in their probing

TABLE 4.1: Regression analysis of the attachment level measurements taken at each bimonthly visit at each site in patient and control groups. Sites were classified according to trends revealed by regression analysis of these measurements into: sites becoming significantly deeper, becoming significantly shallower, and "not changing" sites. The criterion for "not changing" sites was that a trend toward deepening or becoming shallower at that site was not detected over one year at $P<0.05$..

Site Group	Subject Group	No. of Subjects	No. of Sites	Percentage of Sites	Attachment Level Change (mm) Mean ± SE
Becoming Deeper	Control	2(8%)	2	1.4	0.20±0.04
	Patient	25(46.2%)	50	15.3	0.42±0.03
Becoming Shallower	Control	3(12%)	3	2	0.22±0.02
	Patient	7(13%)	8	2.7	0.39±0.02
"Not Changing"	Control	20(8%)	145	96.6	0.13±0.01
	Patient	22(39.8%)	266	82.1	0.12±0.01

attachment and these were occurred in three subjects, while 145(96.6%) sites showed no significant change over the period of study.

4.1.2 Incidence of attachment level change during two months intervals between each successive visits determined by a safety threshold method

A safety threshold method described earlier (2.7.1) using a cut off point of equal to or >2mm and equal to or >1.5mm was used to determine the change in attachment level at each periodontal site in the patient and control groups respectively, between each pair of successive visits and between the first visit and the final visit (over one year). Another less stringent criterion using a safety threshold of 1.5mm was used to determine the attachment level change at each periodontal sites in patient group only.

On the basis of these safety thresholds, the periodontal sites were classified into three categories which were loser, stable and improved sites (2.7.2). The number and percentage, mean and standard error (SE) of attachment level change for each category for patient and control groups is shown in Table 4.2 and Table 4.3.

In the patient group, when a safety threshold of 2mm was used, The percentage of loser sites which lost attachment level equal to or >2mm over each pair of successive visits ranged from 3.2%-7.4%, while 22.4% of the total sites studied became losers during one year. The percentage of

improved sites which gained equal to or >2mm ranged from 1.9-2.9% over each pair of successive visits while it was 0.7% for all the sites studied over one year. The number of subjects who showed attachment loss equal to or > 2mm over each pair of successive visits ranged from 10(18.5%) to 20 subjects(37%), while 36 subjects (66.7%) showed loser sites in their attachment level at this threshold over one year. The mean attachment loss for loser sites between each pair of successive visits ranged from 2.04-2.48mm, while the mean attachment loss for all the loser sites over one year was 2.5mm.

When a less stringent criterion (1.5mm) to determine attachment level change was used, the incidence of loser sites for each pair of successive visits became higher approximately at 30%, while only two more sites classified as loser over a one year period (Table 4.3).

In the control group, using a safety threshold of 1.5mm to determine attachment level change, the percentage for loser sites was very small ranging from 0-2% with a mean attachment loss ranged from 1.5-2mm. The percentage of improved sites was very small ranged from 0-1.33% with a mean of attachment gain ranged from 1.50-2mm. It was obvious from the results (Table 4.3) that in the control group only very few sites for each pair of successive visits and over one year showed attachment level change at this threshold. Thus no further analysis of the data was worth carrying out on the control group using site as an experimental anaylsis.

TABLE 4.2: Classification of attachment level changes per site in patient group between each two successive visits and over one year (visit 1-visit 6), using a safety threshold of 2 mm. The sites were classified into loser, stable and improved sites, and their numbers, percentage as well as mean attachment level change, are shown.

Visits	Classified Sites	No. of Sites	Percentage	Attachment Level Change Mean ± SE	No. of Loser Subjects
Visit 1 -Visit 2	Loser	24	7.4	-2.04±0.13	17
	Stable	293	90.4	-0.01±0.04	
	Improved	7	2.2	+2.24±0.13	
Visit 2 -Visit 3	Loser	14	4.3	-2.23±0.10	11
	Stable	301	92.9	-0.09±0.04	
	Improved	9	2.8	+2.28±0.21	
Visit 3 -Visit 4	Loser	16	4.9	-2.39±0.10	16
	Stable	302	93.20	-0.09±0.04	
	Improved	6	1.9	±2.47±0.20	
Visit 4 -Visit 5	Loser	10	3.2	-2.46±0.14	10
	Stable	293	93.9	-0.07±0.04	
	Improved	9	2.9	+2.28±0.13	
Visit 5 -Visit 6	Loser	23	7.4	-2.48±0.13	20
	Stable	283	90.7	-0.12±0.04	
	Improved	6	1.9	+2.42±0.28	
Visit 1 -Visit 6	Loser	70	22.4	-2.50±0.10	36
	Stable	240	76.9	-0.34±0.04	
	Improved	2	0.7	+3.00±0.32	

TABLE 4.3: Classification of attachment level change per site in both patient and control groups for each two successive visits, using a safety threshold of 1.5mm. The sites were classified into loser, stable and improved sites. The number, percentage as well as mean attachment level change for each site group are shown.

		Loser Sites			Stable Sites			Improved Sites		
Visit	Group	N	%	Mean±SE	N	%	Mean±SE	N	%	Mean±SE
1-2	Control	0	0	0	150	100	0.1±0.04	0	0	0
	Patient	37	11.4	-2.10±0.1	279	86.1	-0.3±0.04	8	2.5	2.2±0.1
2-3	Control	2	1.3	-2.00±0.3	148	98.7	-0.6±0.05	0	0	0
	Patient	26	8.0	-1.91±0.1	287	88.6	-0.4±0.04	11	3.4	2.4±0.2
3-4	Control	3	2.0	-1.75±0.3	146	97.3	-0.3±0.04	1	0.7	1.7±0.0
	Patient	27	8.3	-2.07±0.1	288	88.9	-0.6±0.04	9	2.8	2.2±0.2
4-5	Control	3	2.0	-1.75±0.1	145	96.7	-0.2±0.04	2	1.3	2.0±0.0
	Patient	18	5.5	-2.10±0.1	283	90.7	-0.4±0.04	11	3.4	2.2±0.1
5-6	Control	1	0.7	-1.50±0.0	149	99.3	-0.3±0.04	0	0	0
	Patient	32	9.9	-2.22±0.1	271	86.7	-0.1±0.04	9	2.8	2.2±0.2
1-6	Control	3	2.0	-1.67±0.1	145	96.7	0.2±0.03	2	1.3	1.5±0.0
	Patient	73	23.4	-2.45±0.1	237	76.0	-0.3±0.40	2	0.7	3.0±0.3

4.2 Clinical, microbiological and laboratory findings using the subject as an experimental unit of analysis

Two groups of subjects were used in our study, these were the patient group and the control group.

The patient group consisted of 54 patients who were on maintenance periodontal therapy at 1-3 months recall system. Six sites were selected from each patient (2.2). A total of 324 sites from all patients were studied. The control group consisted of 25 healthy dental students with no history of periodontal disease at selection time and they had good oral hygiene and a healthy periodontium. A total of 150 sites from all the controls were selected for our study. The purpose for selecting these two groups, was to investigate the clinical parameters and CRP levels and their association with attachment level change among these two groups.

In this study, the clinical parameters (PI, GI, BOP, PD, GCF volume), microbiological data (percentages of coccoid cells, other, motile rods and spirochaetes) and serum and saliva CRP were investigated using subject as an experimental unit. The mean of attachment level measurements for each subject at each visit, was calculated by averaging all the values at all six sites within each subject. This was the subject mean for attachment level measurement. In a similar way, the subject mean for pocket depth, GCF volume, and each of bacterial morphotypes at the

initial visit of each pair of successive visits was calculated. The subject mean of each variable at the initial visit was used as a baseline subject mean for the subsequent (recall) visit for each pair of successive visits. For the study over one year, the recorded data at first visit was considered the baseline to the final visit (sixth visit).

The mean attachment level change for each subject over each pair of the successive visits (and over one year) was calculated by subtracting the subject mean of attachment level measurement at the subsequent visit from the attachment level measurement at the initial visit.

Subjects were ranked for their mean attachment level changes over each pair of successive visits (and over one year, between first and sixth visit).

The subject mean for each variable (except PI, GI and BOP) at the initial visit of each pair of successive visits (and at the first visit with relation to sixth visit) was also ranked. Spearman rank correlations using the ranked subject mean for attachment level change and each of the above variables was mainly used in our study when subject was considered as an experimental unit of analysis.

The exception to this, was the study on serum and salivary CRP and their association with attachment level change where no rank system was used.

For discrete variables, PI , GI and BOP, their scores were compared with attachment level change using a Chi-square test.

4.2.1 Clinical findings in the patient and control groups

The clinical parameters for patient and control groups are shown in Table 4.4. This included the mean +_ standard error of pocket depth and attachment level measurements recorded using the stent technique (2.2) at each visit in both the control group and patient group. The data showed that both pocket depth and probing attachment level measurements are higher for the patient group than the control group at all visits. The mean pocket depth for patient group was >3.5mm at all the visits, and did not significantly change during the whole period.

The differences in attachment level for all the subjects (in each group) was averaged, and this produced a mean attachment level change per subject in each group, and this is shown in Table 4.5. It was found that the probing attachment level in the patient group was deeper than in the control group at each visit. The mean attachment level loss during one year (annual attachment level loss) in the patient group was 0.42mm and was higher than in the control group (0.08mm).

The attachment level change per subject over each pair of successive visits, and over one year was investigated for their correlation later on (4.2.4.3) with the change in serum and salivary C-reactive protein (CRP).

TABLE 4.4: Mean and standard deviation (SD) of pocket depth (PD) and attachment level measurement (ALM) per subject at each visit in patient and control groups.

Visit	Group	No of Subjects	PD (mm) Mean ± SD	ALM (mm) Mean ± SD
1	Control	25	1.98 ± 0.27	4.51 ± 0.41
	Patient	54	3.53 ± 0.92	6.18 ± 1.01
2	Control	25	1.98 ± 0.25	4.51 ± 0.46
	Patient	54	3.61 ± 0.91	6.39 ± 1.13
3	Control	25	1.97 ± 0.30	4.59 ± 0.41
	Patient	54	3.66 ± 0.41	6.50 ± 1.16
4	Control	25	2.04 ± 0.30	4.65 ± 0.50
	Patient	54	3.70 ± 0.95	6.66 ± 1.23
5	Control	25	2.13 ± 0.25	4.64 ± 0.43
	Patient	52	3.78 ± 0.96	6.77 ± 1.18
6	Control	25	2.15 ± 0.34	4.68 ± 0.46
	Patient	52	4.04 ± 0.99	7.02 ± 1.24

TABLE 4.5: Mean difference in attachment level measurement per subject between each two successive visits and over one year in control and patient groups.

Visits	Subject Group	Number	Mean difference in in attachment level (mm)
Visit 1-	Control	25	0.008
Visit 2	Patient	54	-0.211
Visit 2-	Control	25	-0.087
Visit 3	Patient	54	-0.105
Visit 3-	Control	25	-0.055
Visit 4	Patient	54	-0.160
Visit 4-	Control	25	0.009
Visit 5	Patient	52	-0.111
Visit 5-	Control	25	-0.039
Visit 6	Patient	52	-0.244
Visit 1-	Control	25	-0.080
Visit 6	Patient	52	-0.420

4.2.2 Association of attachment level change with clinical parameters (PI, GI, BOP, PD) and GCF volume in patient and control groups

The purpose of this study was to find if there was any correlation between attachment level change over each pair of successive visits (and over one year), with the clinical parameters (PI, GI, BOP, PD) and GCF volume. In other words to find if any of these parameters could be associated with or predict disease activity as measured by attachment loss per subject. This study was based on using the subject as an experimental unit (2.7.3).

The subject mean for the attachment level change over each pair of successive visits, and for pocket depth and GCF volume, recorded at the initial visit of each two successive visits, was ranked. Spearman rank correlation was used to test the association of attachment level change with these two clinical parameters in each group, and the results are shown in Table 4.6. No significant correlation was found between either the pocket depth or GCF volume with the attachment level change over any pair of successive visits or over one year in control group. In the patient group, a significant correlation ($r = 0.363$ at $P<0.05$) was found between GCF volume and attachment level change only over one year. For pocket depth, a positive significant correlation ($r=0.290$ at $P<0.05$) was only found with attachment level change for the successive visits 5-6.

TABLE 4.6: Spearman rank correlation test between attachment level change over each two successive visits (and over one year) with pocket depth (PD) and gingival crevicular fluid (GCF) volume at the initial visit in both patient and control groups.

Visit	Group	Number	Spearman rank correlation coefficient (r) Pocket depth (r)	GCF volume (r)
Visit 1- Visit 2	Control	25	0.316	0.360
	Patient	54	0.047	-0.091
Visit 2- Visit 3	Control	25	0.355	0.166
	Patient	54	0.271	0.136
Visit 3- Visit 4	Control	25	0.306	0.231
	Patient	54	-0.149	-0.199
Visit 4- Visit 5	Control	25	0.313	0.410
	Patient	52	0.192	0.189
Visit 5- Visit 6	Control	25	0.308	-0.084
	Patient	52	0.290*	0.022
Visit 1- Visit 6	Control	25	0.291	0.011
	Patient	52	0.080	-0.363*

* = statistically significant at $P < 0.05$

In the other study, a Chi-square test was used to find if there was any association between the scores of PI, GI, BOP and attachment level change in each group over each prescribed period. As is shown in Table 4.7, no significant association was found between attachment level change and PI and between attachment level change and GI in both the control and patient groups. No significant association was found for attachment level change with BOP in the control group, while BOP was significantly ($P<0.05$) associated with attachment level change for the successive visits 3-4 and 4-5, as well as over one year.

4.2.3 Association of attachment level change with bacterial morphotypes (coccoid, other, motile rods, spirochaetes) in the patient and control groups

The purpose of this study is to find if there was any association between subgingival bacterial morphotypes (coccoid cells, other, motile rods and spirochaetes) with the attachment level changes per subject in each of the patient and control groups over each pair of successive visits and during one year. Most interesting is to find if the motile bacteria (motile rods and spirochaetes) are associated with or predict attachment level loss in periodontal patients.

Phase contrast microscopy was used to detect the presence of these bacteria in subgingival plaque collected at each

TABLE 4.7: Chi-square analysis for the association between the attachment level change over each two successive visits (and over one year) with plaque index, gingival index and bleeding on probing at the initial visit in both patient and control groups.

Visit	Group	Number	Chi-square analysis and x^2 value		
			Plaque index x^2 value	Gingival index x^2 value	Bleeding on probing x^2 value
Visit 1- Visit 2	Control	25	0.96	0.02	0.01
	Patient	54	1.85	1.46	1.29
Visit 2- Visit 3	Control	25	0.36	0.03	0.01
	Patient	54	1.21	1.85	0.713
Visit 3- Visit 4	Control	25	1.96	0.02	0.02
	Patient	54	1.84	1.21	4.57*
Visit 4- Visit 5	Control	25	3.24	0.03	0.01
	Patient	52	1.42	0.07	4.97*
Visit 5- Visit 6	Control	25	0.04	0.04	0.01
	Patient	52	0.30	1.80	0.69
Visit 1- Visit 6	Control	25	1.96	0.02	0.02
	Patient	52	0.93	1.20	6.23*

* = statistically significant at $P<0.05$

periodontal site at each visit as early described (2.6.4). The mean percentage of each morphotype was calculated and used for data analysis.

The mean percentage of each morphotype at the initial visit of each pair of successive visits, was ranked for each subject. The attachment level change over each paired successive visits was also ranked for each subject. Spearman rank correlation test (2.7.3) was used to test the association between attachment level change per subject and the subject mean percentage of each bacterial morphotype recorded at the initial visit of each two successive visits. As is shown in Table 4.8,no significant correlation was found between attachment level change and each of these bacterial morphotype in both the patient and control group over any pair of successive visits, and over one year.

TABLE 4.8: Spearman rank correlation test between attachment level change over each two successive visits (and over one year) with coccoid cells, other, motile rods and spirochaetes at the initial visit in both patient and control groups.

			Spearman correlation coefficient (r)			
Visit	Group	Number	Coccoid Cells (r)	Other (r)	Motile Rods (r)	Spirochaetes (r)
Visit 1- Visit 2	Control	25	0.401	-0.0108	-0.065	-0.038
	Patient	54	0.242	-0.220	-0.256	-0.013
Visit 2- Visit 3	Control	25	-0.197	-0.065	0.029	-0.126
	Patient	54	-0.176	0.199	0.262	0.247
Visit 3- Visit 4	Control	25	-0.159	-0.101	0.050	-0.156
	Patient	54	0.268	-0.265	-0.164	0.238
Visit 4- Visit 5	Control	25	0.035	0.336	0.351	0.414
	Patient	52	0.180	0.047	-0.050	-0.104
Visit 5- Visit 6	Control	25	0.115	-0.261	-0.060	-0.074
	Patient	52	-0.164	0.209	0.055	0.223
Visit 1- Visit 6	Control	25	0.162	-0.244	-0.244	-0.181
	Patient	52	0.182	-0.196	0.122	-0.055

4.2.4 Laboratory Findings on serum C-reactive protein (CRP) and salivary CRP in the patient and control groups

The purpose of this study was to find the level of serum and salivary C-reactive protein in both patient and control groups. Serum and saliva samples were collected at each visit from each subject in each group (2.6.1, 2.6.5). The levels of CRP in serum and saliva were determined using the ELISA technique described earlier (3.3.2).

The mean and standard error (SE) of the serum concentration (mg/l) and salivary CRP concentration (ng/ml) for each group at each visit was shown in Table 4.9.

It was found that salivary CRP levels were higher in the patient group compared to control group. The mean salivary CRP concentration at all visits, was 1.045 and 0.175 ng/ml for the patient and control groups respectively. The range of salivary CRP was found to be 0.0-11.97 ng/ml and 0.0-1.89 ng/ml for the patient and control groups respectively. The mean serum CRP concentration at all visits, was 1.752 mg/l and 0.969 mg/l for the patient and control groups respectively. The range for serum CRP was 0.145-20.0 mg/l and 0.140-8.0 mg/l for the patient and control groups respectively.

TABLE 4.9: Levels of serum CRP and saliva CRP in each of the patient and control groups at each visit, and over all visits. The mean and standard error (SE) of CRP levels are shown.

		Serum CRP Concentration (mg/L)		Saliva CRP Concentration (ng/ml)	
Visit	Subject Group	No. of Subjects	Mean ± SE	No. of Subjects	Mean ± SE
1	Control	25	0.999±0.242	25	0.087±0.076
	Patient	54	1.594±0.349	54	0.756±0.232
2	Control	25	1.111±0.269	25	0.098±0.065
	Patient	54	1.010±0.157	53	1.235±0.329
3	Control	25	1.321±0.434	25	0.134±0.060
	Patient	50	1.588±0.353	54	1.247±0.276
4	Control	25	1.361±0.356	25	0.235±0.064
	Patient	50	2.397±0.475	54	1.085±0.240
5	Control	25	0.541±0.096	25	0.204±0.076
	Patient	48	1.741±0.570	51	0.789±0.150
6	Control	25	0.578±0.159	25	0.299±0.076
	Patient	51	2.276±0.540	52	1.161±0.135
All Visits	Control	150	0.969±0.11	150	0.175±0.031
	Patient	307	1.752±0.650	318	1.045±0.107

4.2.4.1 Correlation of serum and saliva CRP levels in each subject group

The aim of this study was to find if there is a correlation between the level of serum and salivary CRP in each group at each visit.

Pearson correlation analysis was applied to test this correlation, and the result are shown in Table 4.10.

At most visits, no significant correlation was found between the levels of serum CRP and saliva CRP in each subject group.

4.2.4.2 Comparison between control and patient groups for their serum and salivary CRP levels

The aim of this study was to compare the patient group and the control group for their levels of salivary CRP and serum CRP. Using analysis of variance, the levels of salivary CRP and also the levels of serum CRP, between patient and control groups at each visit was compared.

As shown in Table 4.11, the serum CRP level was found to be significantly higher ($P<0.05$) in the patient group compared to its level in control group at only two visits. It was also found that the levels of salivary CRP in the patient group were significantly higher ($P<0.01$) than in the control group at each visit.

TABLE 4.10: Pearson correlation test between serum CRP concentration (mg/L) and saliva CRP concentration (ng/ml) in each of the patient and control group at each visit. Pearson correlation coefficient (r) and P-value are shown.

Pearson Correlation Test between Serum CRP and Saliva CRP

Visit	Group	Number	Correlation Coefficient	P Value
1	Patient	54	0.080	0.277
	Control	25	0.036	0.431
2	Patient	53	0.094	0.251
	Control	25	0.164	0.216
3	Patient	50	0.010	0.488
	Control	25	0.097	0.323
4	Patient	50	0.224	0.059
	Control	25	0.410	0.020
5	Patient	51	0.420	0.002
	Control	25	0.787	0.001
6	Patient	48	0.480	0.002
	Control	25	0.060	0.382

TABLE 4.11: t-test analysis of the differences in serum CRP levels and saliva CRP levels between patient and control groups at each visit.

Visit	t-test for saliva CRP Difference P-value	t-test for serum CRP Difference P-Value
1	0.008	0.172
2	0.001	0.76
3	0.001	0.63
4	0.000	0.06
5	0.001	0.02
6	0.012	0.01

4.2.4.3 Association of serum CRP and saliva CRP with attachment level changes in each group

In this study, the levels of serum and salivary CRP was tested for their association with attachment level changes per subject (in each group) over each two successive visits and over one year.

Regression analysis using Pearson correlation coefficient was used to test if there is an association between the attachment level change per subject with the change in salivary and serum CRP, over each pair of successive visits and over one year in each subject group.

As shown in Table 4.12, no significant correlation was found between attachment level change and the change in serum or salivary CRP in each group at any occasion.

TABLE 4.12 Regression analysis for attachment level change and the change in the saliva CRP concentration and serum CRP concentration in each group over each two successive visits, and over one year. Pearson correlation coefficient and P value are shown.

		Pearson correlation (r) between attachment level change and saliva CRP change			Pearson correlation (r) between attachment level change and serum CRP change		
Visit	Group	Number	r	P-value	Number	r	P-value
1-2	Control	25	0.065	NS	25	-0.236	NS
	Patient	54	0.015	NS	54	-0.146	NS
2-3	Control	25	0.131	NS	25	0.031	NS
	Patient	53	-0.011	NS	53	-0.129	NS
3-4	Control	25	0.229	NS	25	-0.164	NS
	Patient	50	0.066	NS	50	-0.190	NS
4-5	Control	25	0.041	NS	25	0.22	NS
	Patient	52	0.161	NS	52	-0.138	NS
5-6	Control	25	0.200	NS	25	0.155	NS
	Patient	52	0.067	NS	52	0.169	NS
1-6	Control	25	-0.20	NS	25	-0.2	NS
	Patient	50	-0.42	NS	50	-0.14	NS

NS = not significant at $P<0.05$

4.3 Clinical, microbiologic and laboratory findings in the patient and control groups using the site as an experimental unit of analysis

In this study, the site was used as an experimental unit of analysis in each of patient and control groups as follows.

4.3.1 Clinical findings (PI, GI, BOP, PD, attachment level measurement) and GCF volume for periodontal sites in patient and control groups

The clinical data for PI, GI, BOP, PD and attachment level measurement as well as GCF was collected as described earlier for each site in each group at each visit. A total of 324 sites and 150 sites were used at each visit in this study for the patient and control groups respectively. The details including mean and standard error of each variable for all periodontal sites in each group and each visit are shown in Tables 4.13, 4.14, and Table 4.20 for GCF volume. The attachment level was measured relatively to the fixed reference point on the stent and it was considered as a relative attachment level measurement. The BOP was expressed as a percentage of sites that bled on probing at each visit.

As shown from the results, the mean PD was ranged from 3.53 to 4.04mm for periodontal sites in the patient group while it was ranged from 1.98 to 2.15mm for all the periodontal

sites in the control group. The mean pocket depth for all the sites for all the visits in the patient group was 3.72mm and higher than the control group which was 2mm. The mean GCF volume (Table 4.20) for all sites ranged from 0.07 to 0.10uL and from 0.242 to 0.31 uL for all periodontal sites in the control and patient group respectively. The mean GCF volume for all sites at all visits in the patient group was 0.279uL and higher than in the control group which was 0.081uL.

The frequency of BOP ranged from 41-49% and from 4-8% for all the sites in the patient and control groups respectively. The mean scores of both PI and GI for all periodontal sites in the patient group for all visits was 2.28 and 0.30 respectively.

The frequency distributions of bleeding sites, plaque index and gingival index scores at the initial visit of each pair of successive visits for the patient group are shown in Figure 4.1 for bleeding sites; Figures 4.2a,b,c,d,e,f for plaque index scores, and Figures 4.3a,b,c,d,e,f for gingival index scores. As is shown higher frequency of bleeding sites were found for loser sites than stable sites on all occasions except for the successive visits 5-6. It was also found that high plaque scores (2-3) were found for both loser and stable site on most occasions. Low gingival scores were found for both loser and stable site groups as well as for all sites together on all occasions.

TABLE 4.13: Clinical data of all sites in the patient group at each visit. The mean and standard error (SE) of the plaque index (PI), gingival index (GI), pocket depth (PD) and attachment level measurement (ALM) are shown. Bleeding on probing (BOP) is expressed in frequency percentage.

Mean ± SE of Clinical Data

Visit	No. of Sites	PI Mean ± SE	GI Mean ± SE	BOP %	PD(mm) Mean ± SE	ALM (mm) Mean ± SE
1	324	2.38±0.05	0.50±0.05	43	3.53±0.88	6.1±0.79
2	324	2.24±0.05	0.23±0.04	41	3.61±0.94	6.4±0.86
3	324	2.29±0.05	0.32±0.05	44	3.60±0.91	6.5±0.86
4	324	2.60±0.05	0.28±0.04	44	3.70±0.92	6.7±0.90
5	312	2.26±0.04	0.20±0.04	44	3.78±0.93	6.8±0.97
6	312	2.30±0.04	0.25±0.05	49	4.04±0.97	7.2±0.98
All Visits	1920	2.28±0.01	0.30±0.02	44	3.72±0.36	6.5±0.45

TABLE 4.14: Mean and standard error (SE) of pocket depth (PD) and attachment level measurement (ALM) for all the sites in the control group at each visit. The frequency of BOP was also shown in frequency percentage.

Visit	No. of Sites	PD Mean ± SE	ALM Mean ± SE	BOP
1	150	1.80±0.59	4.51±0.70	8%
2	150	1.98±0.59	4.51±0.68	4%
3	150	1.97±0.59	4.59±0.68	8%
4	150	2.04±0.58	4.65±0.68	4%
5	150	2.13±0.60	4.64±0.64	8%
6	150	2.15±0.62	4.68±0.64	4%
All Visits	900	2.00±0.25	4.59±0.27	6%

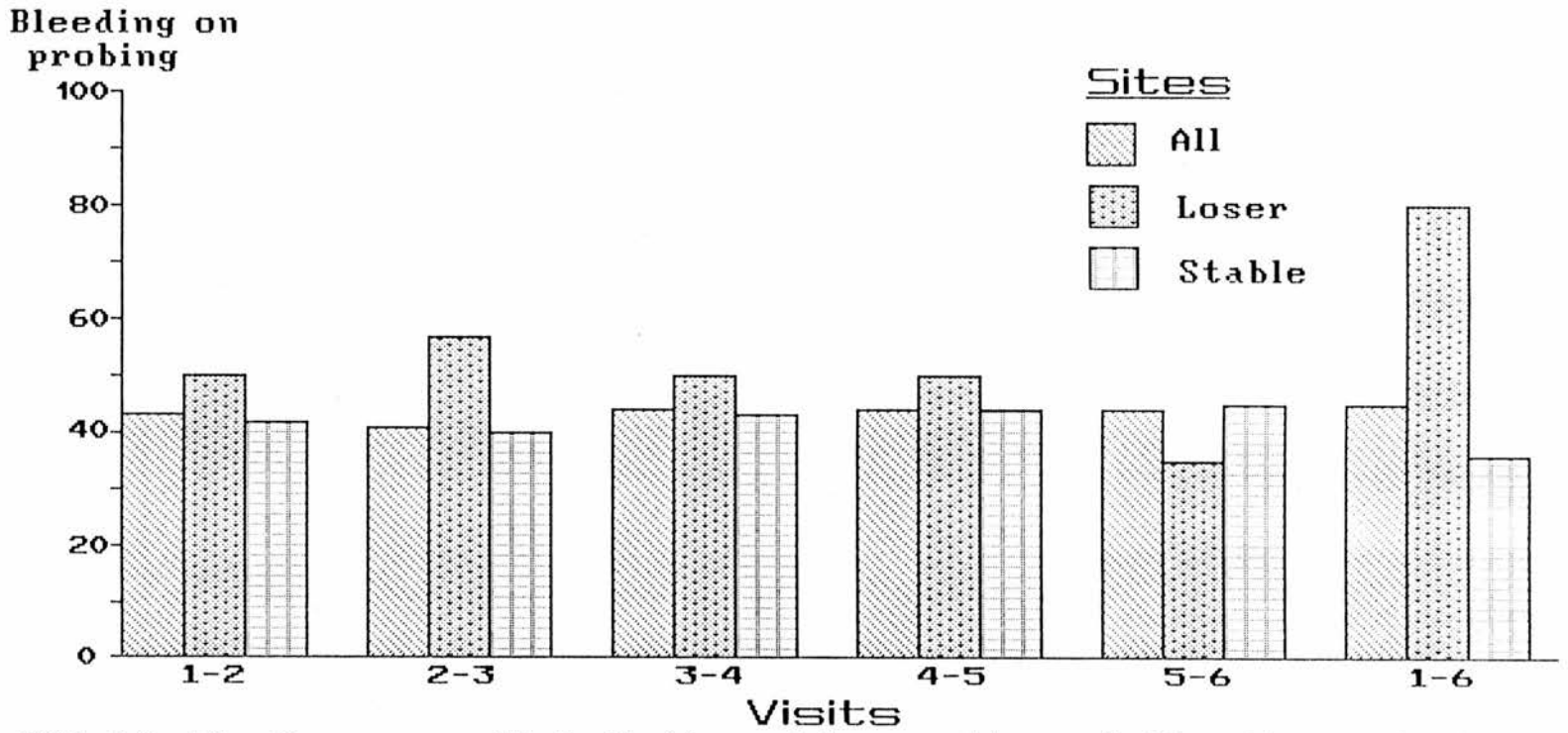


FIG. 4.1 The frequency distribution of loser sites, stable sites and all the sites with bleeding on probing at the initial visit of each pair of successive visits (and over one year).

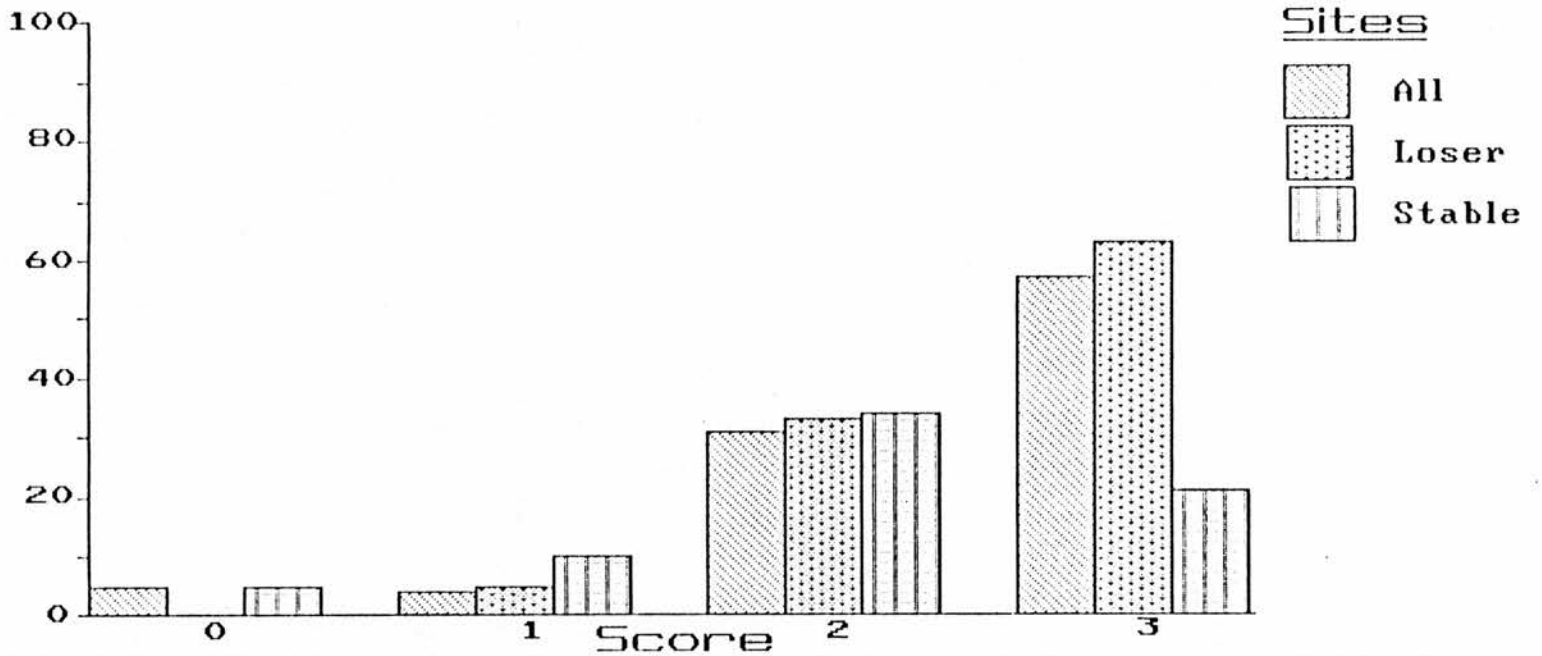


FIG. 4.2.a The frequency distribution of plaque index scores at the loser sites, the stable sites and all the sites in patient group at the initial visit of the successive visits 1 - 2.

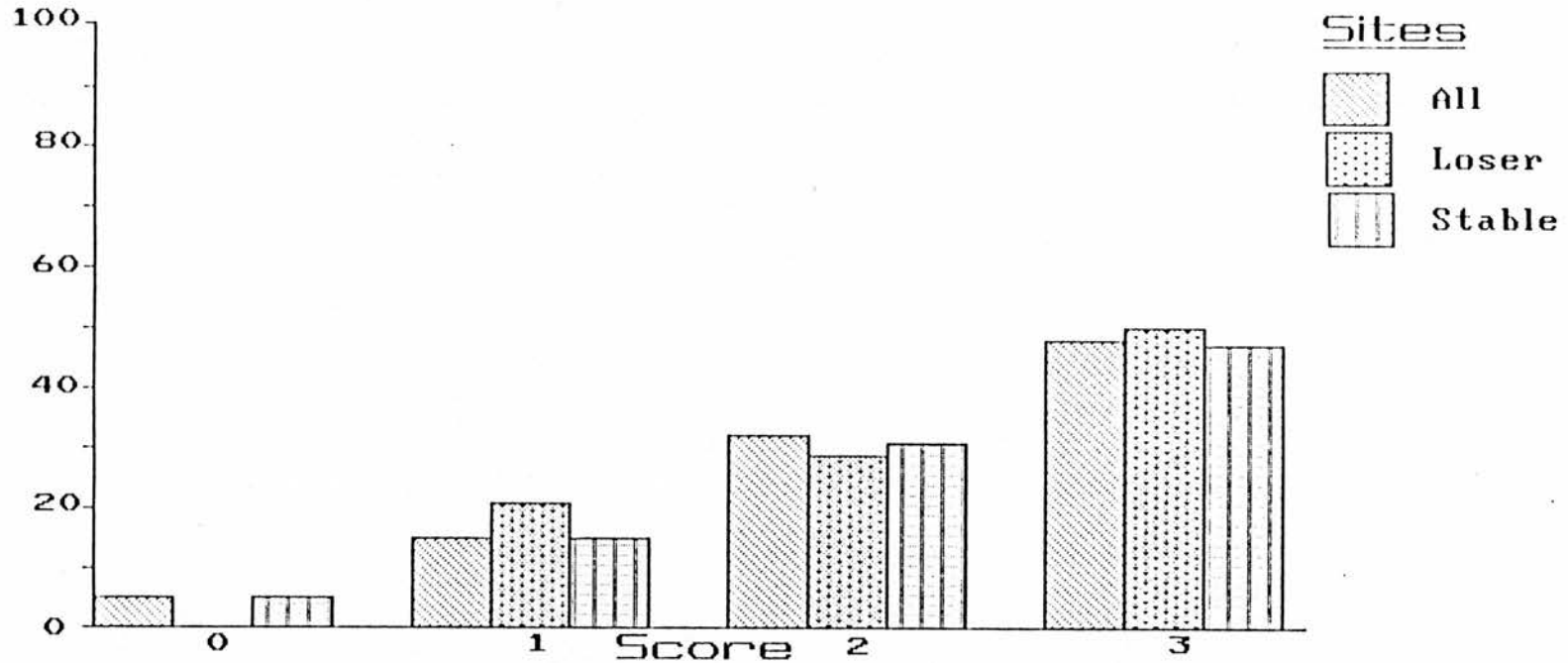


FIG. 4.2.b The frequency distribution of plaque index scores at the loser sites, the stable sites and all the sites in patient group at the initial visit of the successive visits 2 - 3.

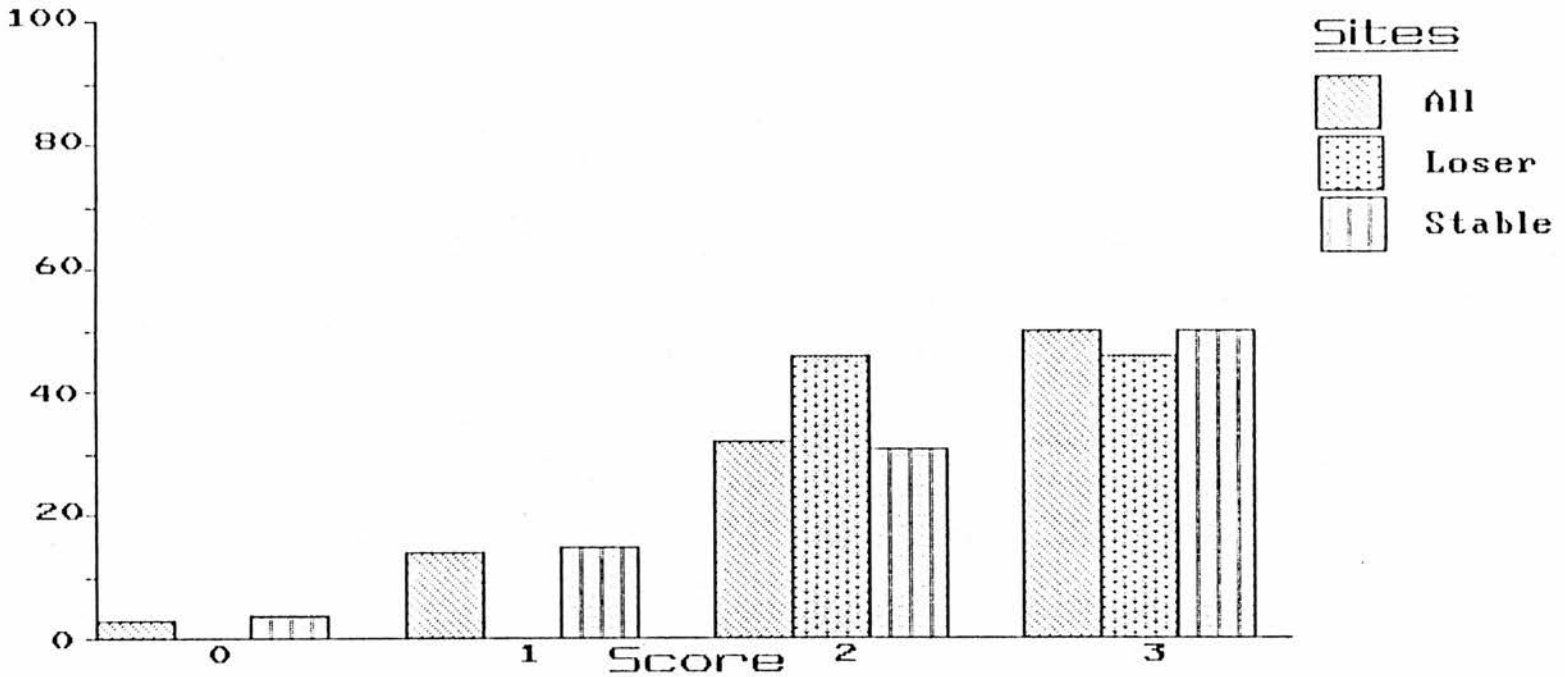


FIG. 4.2.c The frequency distribution of plaque index scores at the loser sites, the stable sites and all the sites in patient group at the initial visit of the successive visits 3 - 4.

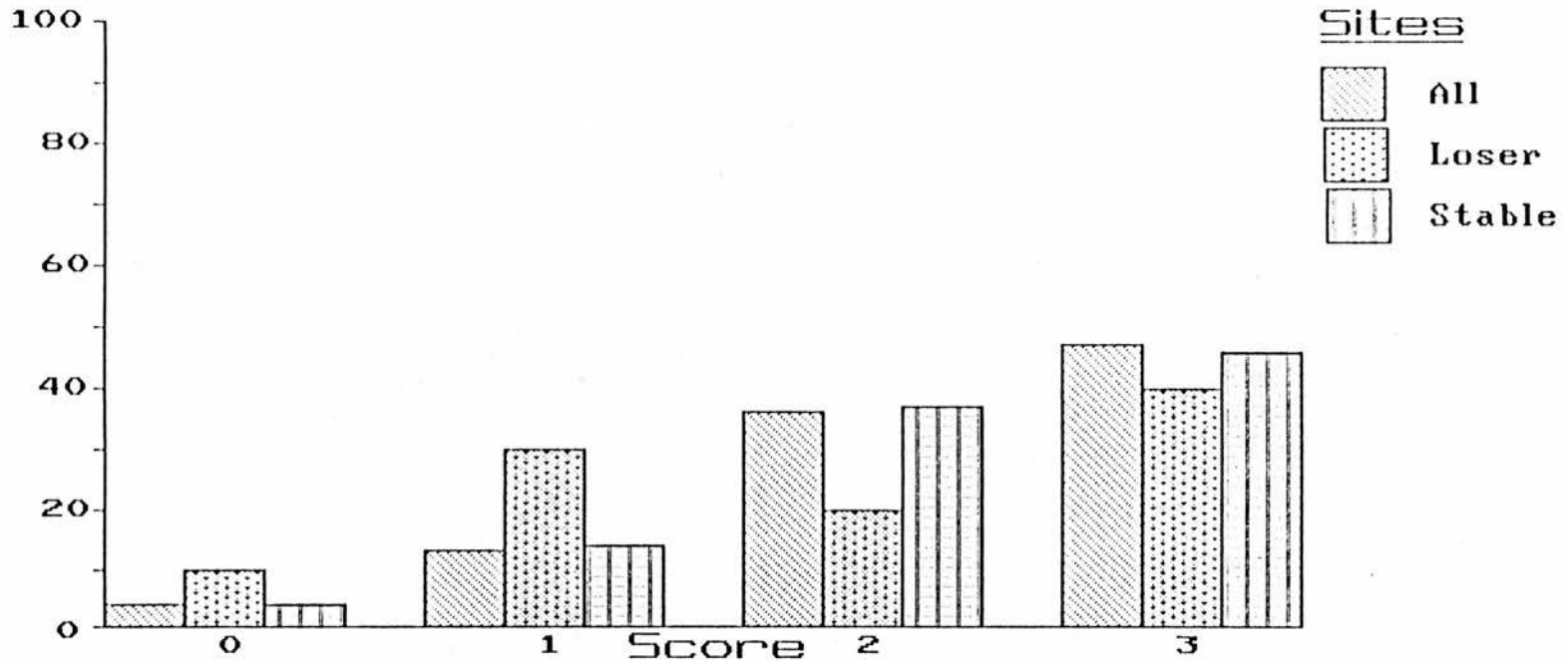


FIG. 4.2.d The frequency distribution of plaque index scores at the loser sites, the stable sites and all the sites in patient group at the initial visit of the successive visits 4 - 5.

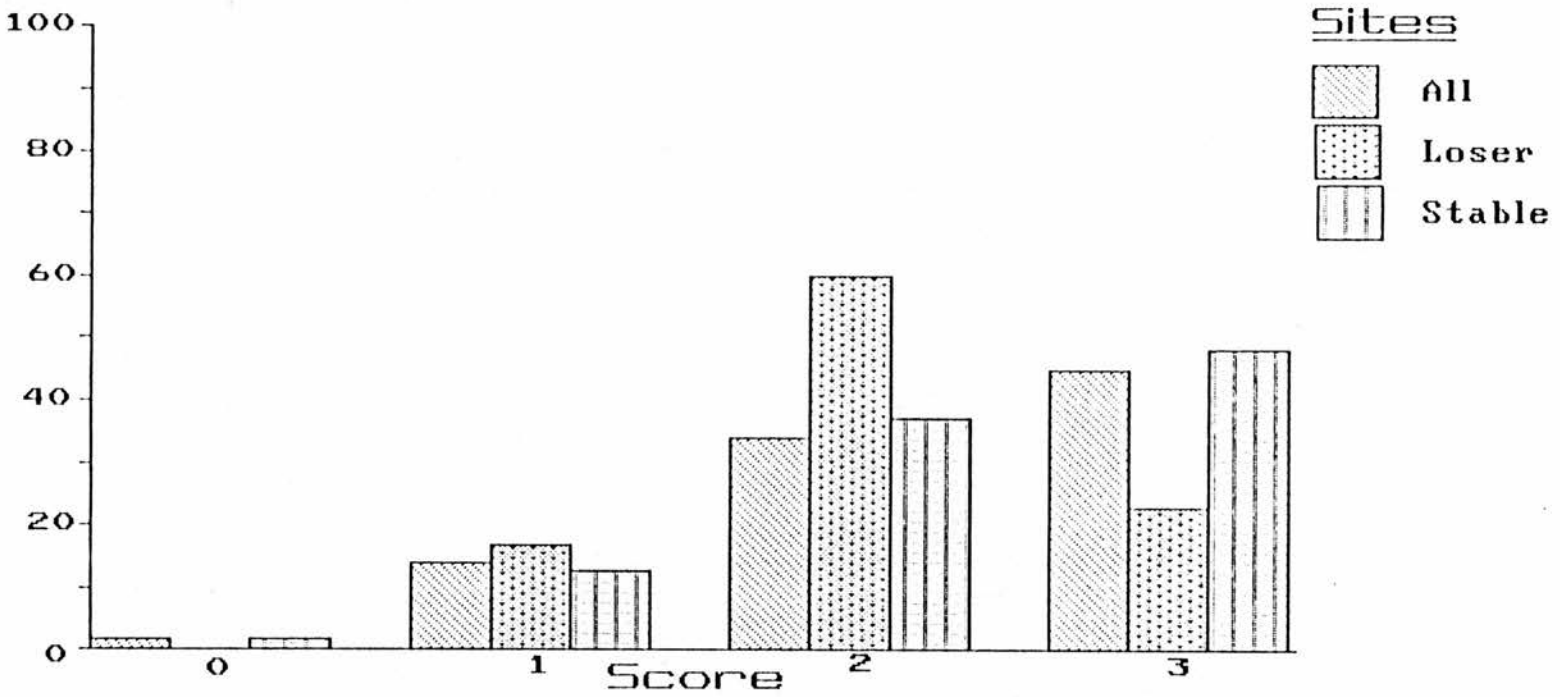


FIG. 4.2.e The frequency distribution of plaque index scores at the loser sites, the stable sites and all the sites in patient group at the initial visit of the successive visits 5 - 6.

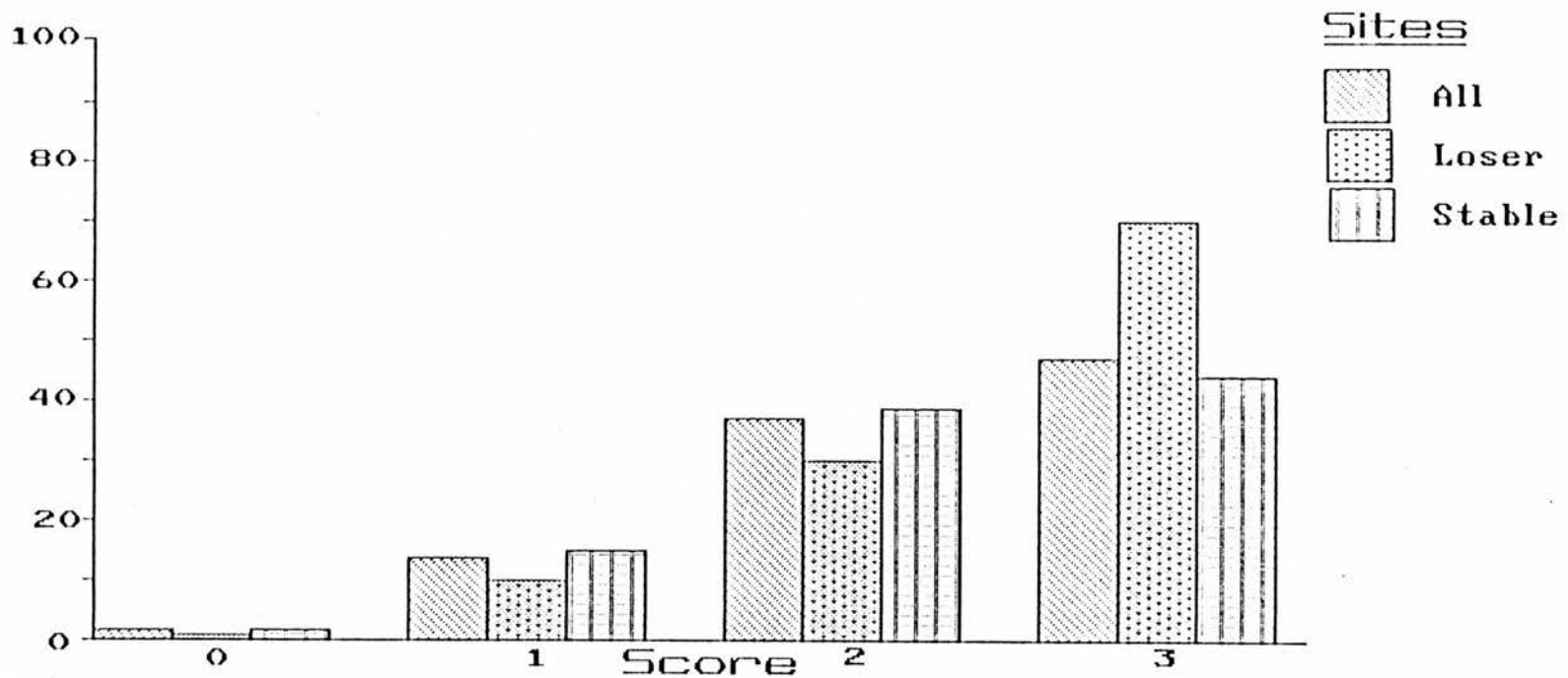


FIG. 4.2.f The frequency distribution of plaque index scores at the loser sites, the stable sites and all the sites in patient group at the initial visit of the one year period (visit 1 - visit 6).

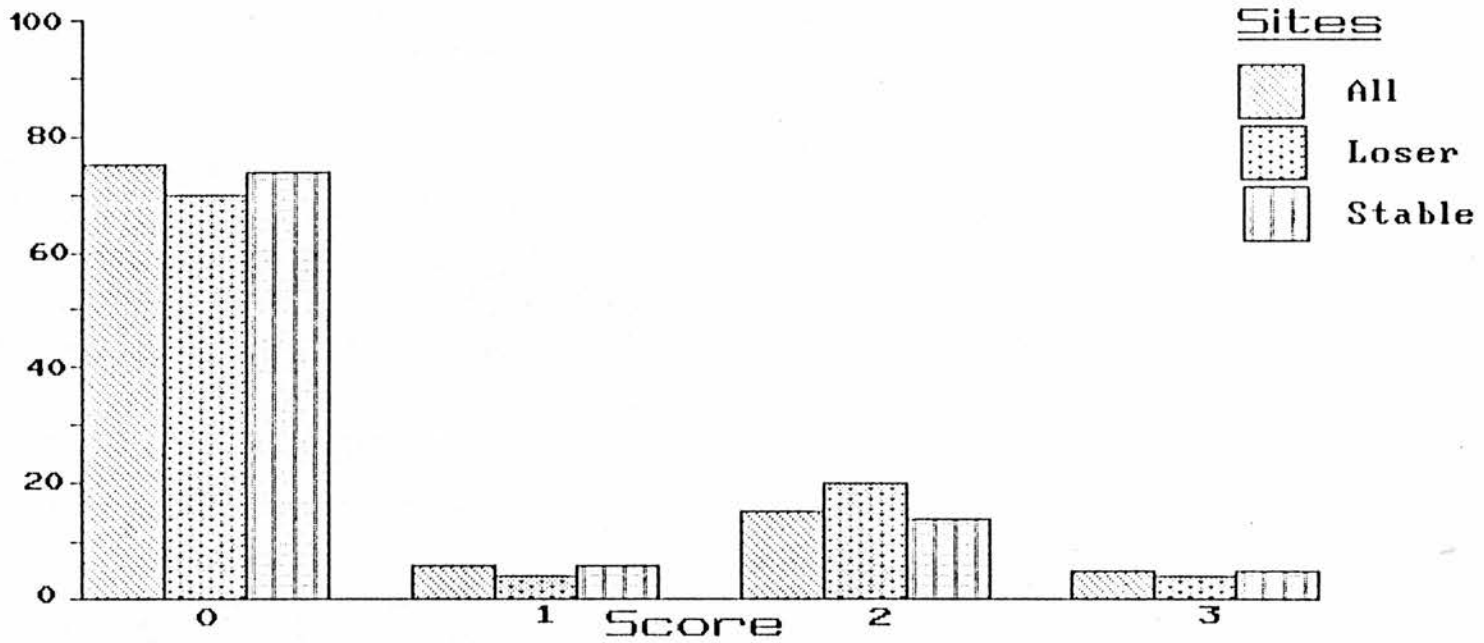


FIG. 4.3.a The frequency distribution of gingival index at the loser sites, the stable sites and all the sites at the initial visit of the successive visits 1 - 2.

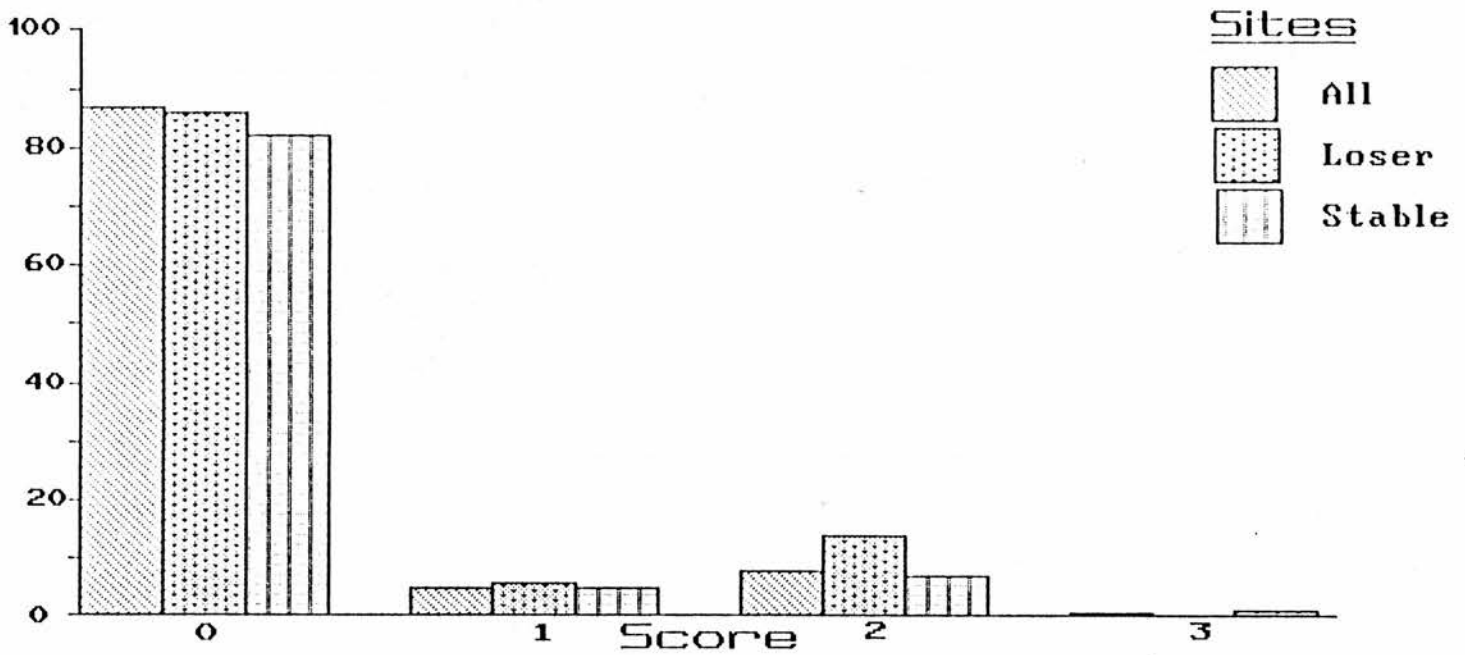


FIG. 4.3.b The frequency distribution of gingival index at the loser sites, the stable sites and all the sites at the initial visit of the successive visits 2 - 3.

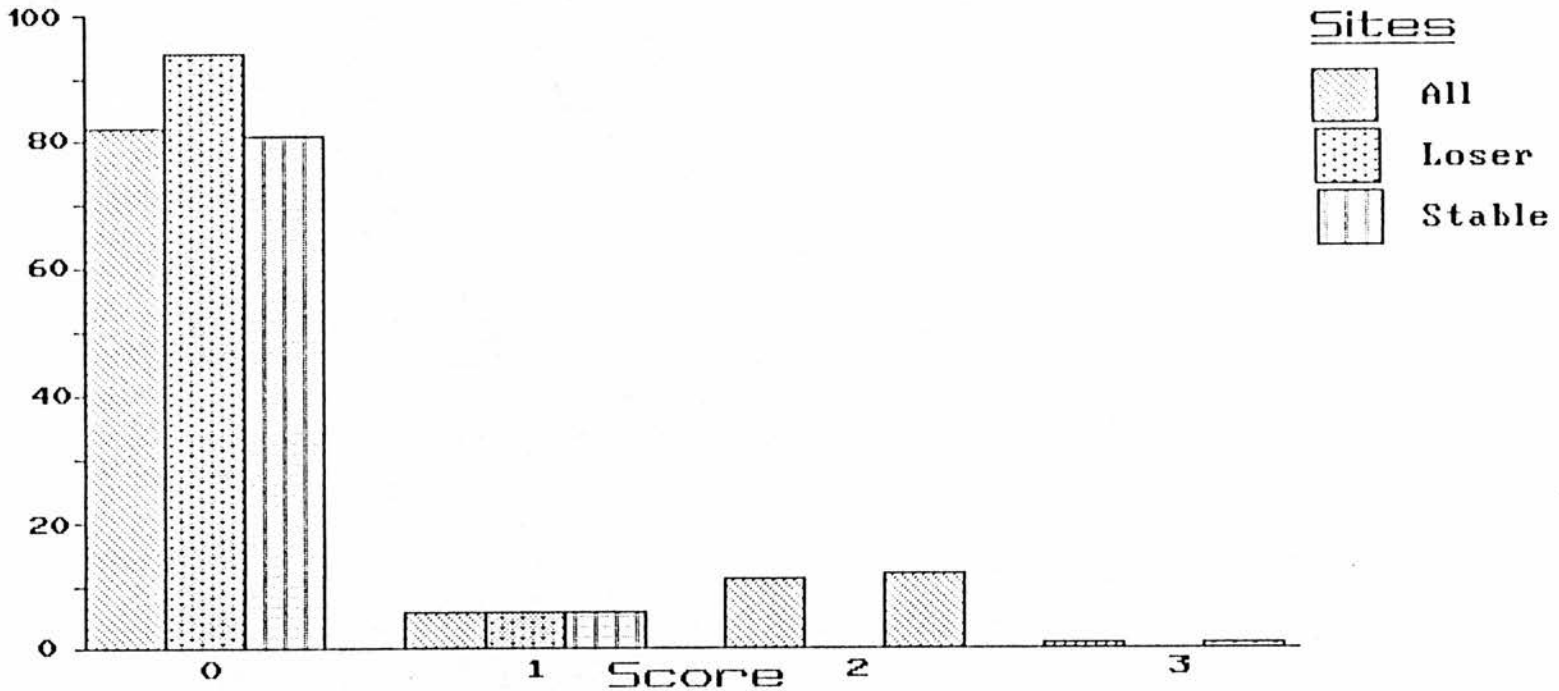


FIG. 4.3.c The frequency distribution of gingival index at the loser sites, the stable sites and all the sites at the initial visit of the successive visits 3 - 4.

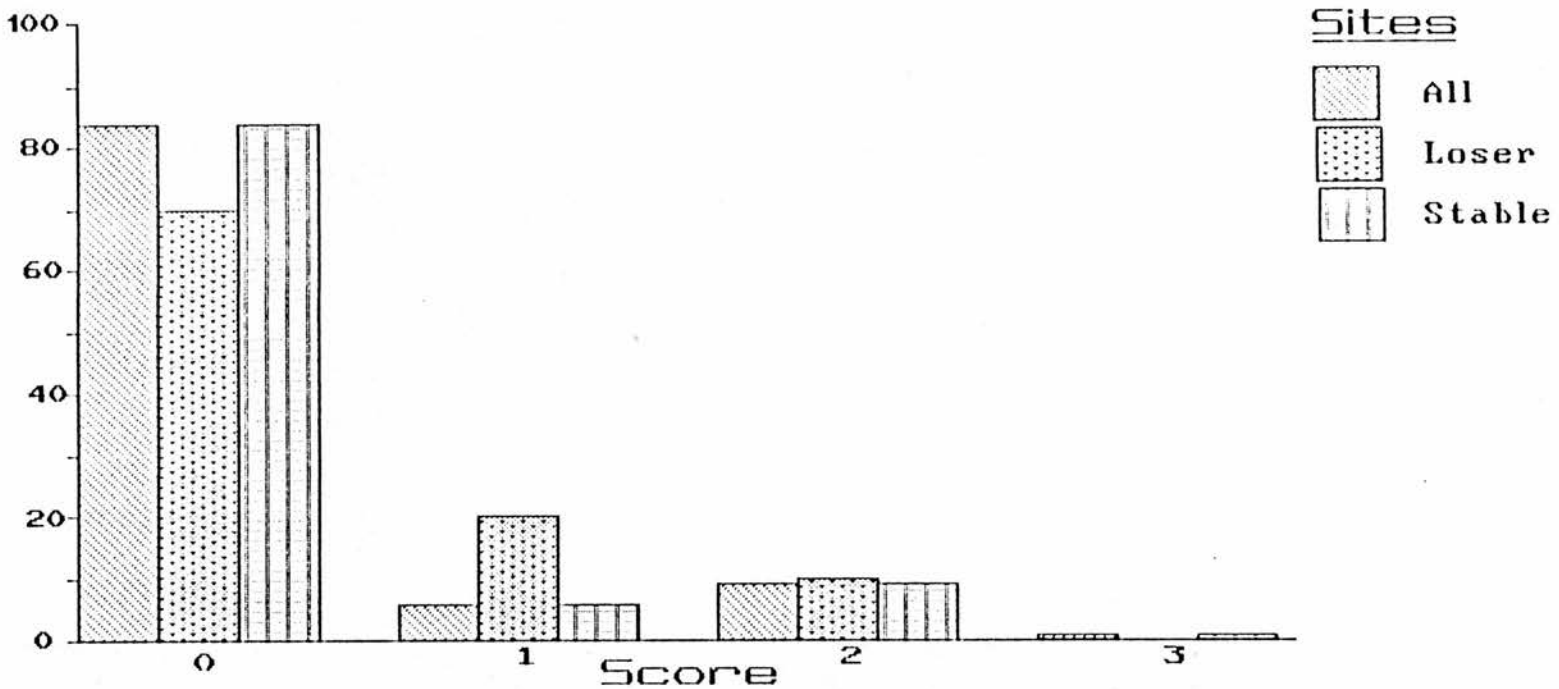


FIG. 4.3.d The frequency distribution of gingival index at the loser sites, the stable sites and all the sites at the initial visit of the successive visits 4 - 5.

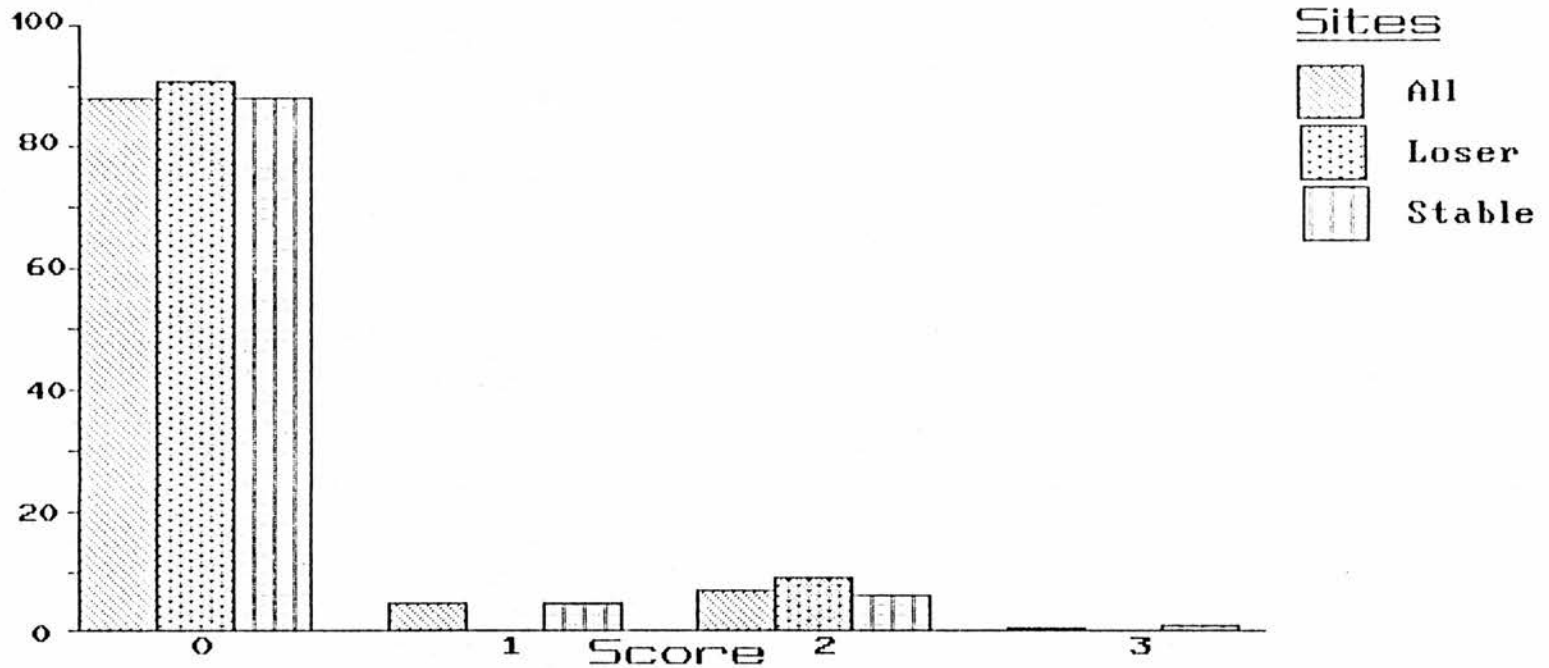


FIG. 4.3.e The frequency distribution of gingival index at the loser sites, the stable sites and all the sites at the initial visit of the successive visits 5 - 6.

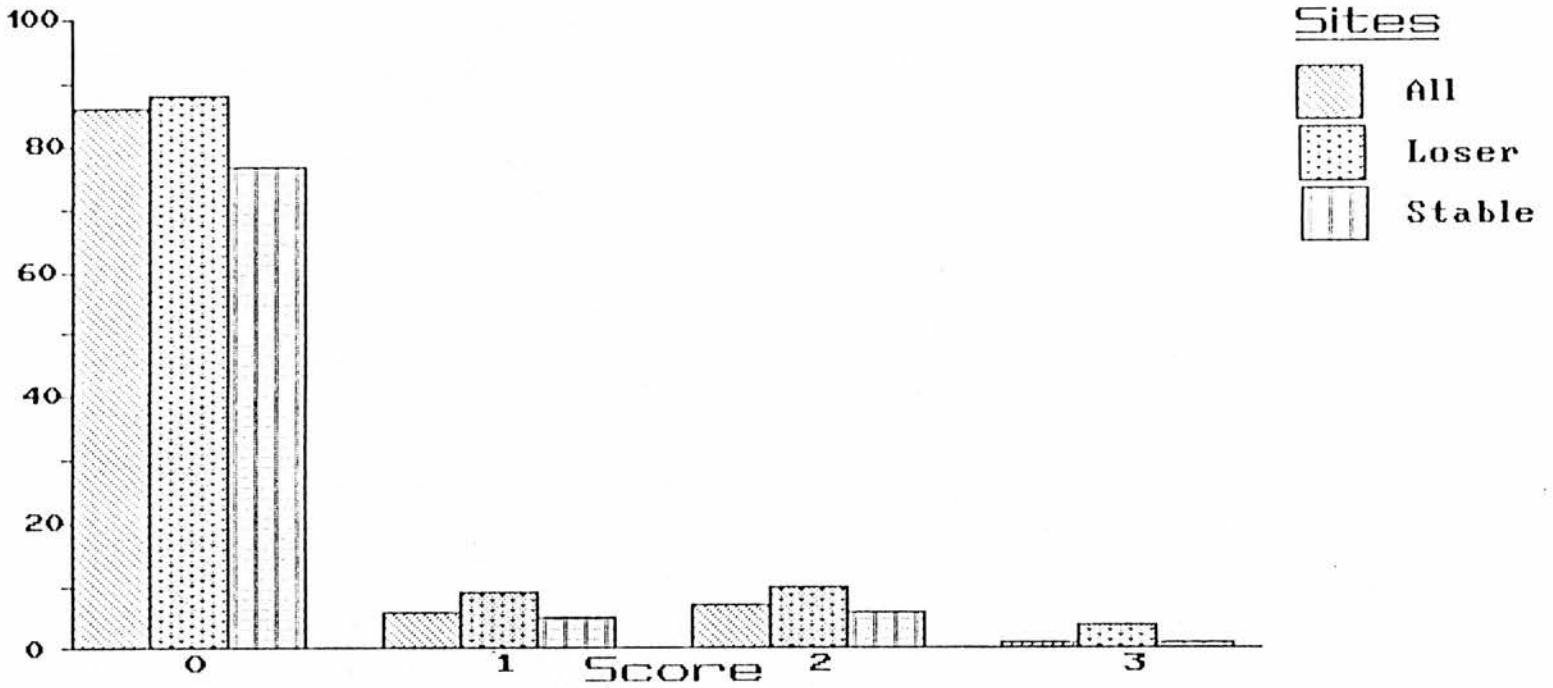


FIG. 4.3.f The frequency distribution of gingival index at the loser sites, the stable sites and all the sites at the initial visit of the one year period (visit 1 - visit 6).

4.3.2 Microbiologic findings (coccoid cells, other, motile rods and spirochaetes) in the patient and control groups

The subgingival plaque sampled at each site as described earlier (2.6.3) was analysed individually using phase-contrast microscopy. The mean percentages and standard error (SE) of each bacterial morphotype (coccoid cell, other, motile rods and spirochaetes) for all sites in each group at each visit is shown in Table 4.15. The range of the mean percentage of motile rods for all sites was 2.09-2.97% in the patient group and was higher than that for all the sites in control group (0.05 -0.26%).

The mean percentage of motile rods for all visits was 0.13% and 2.04% for the control and patient groups respectively. The range of mean percentages of spirochaetes for all the sites in patient group was 5.57-6.70% and was higher than that of all the sites in control group (0.23-0.54%). The mean percentage of spirochaetes for all the sites at all visits was 0.40% and 5.77% in the control and patient groups respectively.

TABLE 4.15: Mean ± standard error (SE) of the percentage for coccoid cells, others, motile rods and spirochaetes for all sites in the control and patient groups at each visit.

Visit	Subject Group	No of Sites	Mean ± Standard Error (SE)			
			Coccoid cells	Other	Motile rods	Spirochaetes
Visit 1	Control	150	85.2±1.6	11.5±2.3	0.26±0.06	0.36±0.13
	Patient	324	56.3±1.4	32.4±1.0	2.97±0.33	5.57±0.56
Visit 2	Control	150	73.8±2.8	9.3±1.9	0.09±0.02	0.23±0.09
	Patient	324	54.6±1.5	32.6±1.1	2.49±0.32	5.70±0.51
Visit 3	Control	150	68.3±3.2	9.11±4.1	0.05±0.02	0.54±0.12
	Patient	324	56.0±1.4	34.6±1.0	2.09±0.22	6.10±0.54
Visit 4	Control	150	66.9±3.1	10.1±3.1	0.13±0.07	0.30±0.12
	Patient	324	53.0±1.4	34.1±1.1	2.28±0.25	5.38±0.48
Visit 5	Control	150	59.8±3.1	12.3±1.9	0.04±0.03	0.45±0.13
	Patient	312	51.2±1.6	31.6±1.2	2.16±0.25	6.19±0.63
Visit 6	Control	150	54.9±3.4	9.7±1.7	0.15±0.04	0.53±0.15
	Patient	312	45.7±1.6	37.0±1.3	2.44±0.29	5.65±0.52
All Visits	Control	900	68.2±1.2	10.3±0.3	0.13±0.02	0.40±0.06
	Patient	1920	52.8±0.6	33.8±0.5	2.40±0.12	5.77±0.22

4.3.3 Association of attachment level change per site with clinical and microbiological variables in each site group in the periodontal patients

The aim of this study was to find if there was any association between attachment level change over each two successive visits and over one year with the initial value of each of the clinical and microbiological variables measured at the initial visit of each pair of successive visits. In other words to find if any of these variables at their initial measurement would be associated with attachment level breakdown or predict disease activity over each prescribed period.

All the sites studied in the patient group were classified into the three categories previously mentioned (2.7.2). These were loser, stable and improved sites. The mean and standard error of their attachment level change over each prescribed period, and their number and percentage was already been shown in Table 4.2. Regression analysis using Pearson correlation coefficient and the Spearman rank correlation test using Spearman correlation coefficient were used to test the association between attachment level change over each prescribed period and the clinical variables (pocket depth, GCF volume) and for microbiologic variables (coccoid cells, other, motile rods and spirochaetes) recorded at the initial visit of each pair of successive visits and at the first visit which corresponded to the change over one year.

Chi-square analysis was used to test the association of attachment level change over each prescribed period with the scores of plaque index (PI), gingival index (GI) and bleeding on probing (BOP).

The results for these associations are shown in Tables 4.16-19, where the mean and standard error of the initial value of each variable at each previous visit, the Pearson correlation coefficient and Spearman correlation coefficient are shown.

As was shown from the results (Table 4.16) no significant correlation between attachment level change and pocket depth, was found at almost all occasions for all three groups of sites using both regression analysis and Spearman correlation tests. The only significant ($P<0.05$) correlation for pocket depth with attachment level change found was over the successive visits 4-5 for stable sites.

No significant association was found between attachment level change and GCF volume for any site group at most occasions except for loser sites for the successive visits 5-6.

No significant correlation was found between attachment level change and each of the subgingival bacteria (coccoid cells, other, motile rods) for each site group on any occasion (Table 4.18,19). A significant correlation ($r= 0.464$, at $P < 0.05$) was found with spirochaetes for the loser sites for the successive visits 1-2 only.

No significant correlation was found between attachment level change and plaque index for any site group on any

occasion except the successive visits 5-6 for loser sites (X^2 value= 7.34) (Table 4.17).

For gingival index, it was found that attachment level change associated with significantly low scores of GI for both loser and stable sites at most occasions.

With respect to bleeding on probing and its association with attachment level change, the only significant correlation was found with loser sites over one year,while for stable sites a significant correlation was found for the successive visits 1-2, and 2-3 (Table 4.17).

TABLE 4.16: Regression analysis and Spearman correlation analysis of the association of attachment level change over each successive visits (and over one year) with initial values of pocket depth and GCF volume at the previous visit in each site group. Pearson and Spearman correlation coefficients are shown.

Visit	Site Group	(N)	Initial PD (mm) Mean±SE	Correlation Coefficient Pearson	Correlation Coefficient Spearman	Initial GCF Volume (uL) Mean±SE	Correlation Co-efficient Pearson	Correlation Co-efficient Spearman
1-2	Loser		4.06±0.31	-0.215	-0.102	0.380±0.016	-0.029	0.003
	Stable		3.45±0.10	0.122	0.102	0.253±0.015	-0.052	-0.032
	Improved		5.03±0.60	0.413	0.333	0.420±0.097	-0.078	0.170
2-3	Loser		3.80±0.44	0.166	0.139	0.428±0.064	0.123	0.044
	Stable		3.52±0.10	0.197	0.178	0.250±0.013	-0.003	-0.005
	Improved		6.11±0.55	0.688	0.700	0.572±0.080	0.318	0.413
3-4	Loser		3.86±0.04	0.063	0.029	0.268±0.052	0.084	0.102
	Stable		3.59±0.10	0.029	0.052	0.240±0.012	0.022	-0.004
	Improved		6.80±0.065	0.209	0.000	0.286±0.085	-0.496	0.478
4-5	Loser		4.18±0.52	-0.144	-0.232	0.309±0.079	0.057	-0.355
	Stable		3.63±0.10	0.213*	0,213*	0.278±0.014	0.109	0.116
	Improved		5.50±0.55	-0.176	-0.138	0.287±0.083	-0.323	-0.584
5-6	Loser		3.48±0.34	-0.294	-0.176	0.33±0.050	-0.466*	-0.064
	Stable		3.75±0.10	0.114	0.099	0.269±0.014	-0.033	-0.089
	Improved		6.25±0.66	0.734	0.616	0.290±0.099	0.772	0.154
1-6	Loser		3.72±0.20	-0.071	-0.030	0.339±0.031	-0.175	-0.125
	Stable		3.47±0.10	0.134	0.138	0.249±0.016	0.059	0.027
	Improved		7.00±1.11	0.000	0.000	0.632±0.183	0.000	0.000

* = statistically significant at $P<0.05$

TABLE 4.17: Chi-square analysis of the association of attachment level change over each two successive visits (and over one year) with initial measurements of PI, GI, and BOP in each site group. X^2 value was shown.

		Chi Square Analysis					
		Initial PI	X^2	Initial GI	X^2	Initial BOP	X^2
Visit	Site Group	Mean±SE	Value	Mean±SE	Value	Frequency	Value
Visit 1- Visit 2	Loser	2.58±0.15	1.5	0.58±0.20	4.2*	50	0.0
	Stable	2.30±0.05	0.35	0.50±0.06	68.0*	42	6.3*
	Improved	2.14±0.38	0.00	0.25±0.20	0.0	10	0.0
Visit 2- Visit 3	Loser	2.28±0.24	0.00	0.29±0.22	7.2*	57	0.3
	Stable	2.23±0.06	1.38	0.32±0.04	70.0*	70	11.2*
	Improved	2.61±0.23	0.00	0.67±0.40	0.0	10	0.0
Visit 3- Visit 4	Loser	2.56±0.17	2.00	0.40±0.25	5.1*	50	0.0
	Stable	2.26±0.05	0.03	0.28±0.04	80.0*	43	3.7
	Improved	2.56±0.24	0.00	0.67±0.46	0.0	12	0.0
Visit 4- Visit 5	Loser	2.00±0.32	0.40	0.40±0.25	1.6	50	0.0
	Stable	2.26±0.05	0.61	0.28±0.04	85.0*	44	3.8
	Improved	2.26±0.24	0.00	0.40±0.35	0.0	17	0.0
Visit 5- Visit 6	Loser	2.04±0.17	7.34*	0.17±0.16	15.7*	35	2.1
	Stable	2.27±0.05	0.05	0.20±0.04	86.0*	46	2.7
	Improved	2.26±0.20	0.00	0.35±0.27	0.0	17	0.0
Visit 1- Visit 6	Loser	2.46±0.10	2.05	0.41±0.71	20.6*	80	25.0*
	Stable	2.54±0.05	3.75	0.20±0.40	83.0*	37	3.2
	Improved	2.50±0.05	0.25	0.0±0.00	0.0	0	0.0

* = statistically significant at $P<0.05$

TABLE 4.18: Regression analysis and Spearman correlation test for the association of attachment level change over each two successive visits (and over one year), with the initial value of coccoid cells and others at the previous visit in each site group. Pearson and Spearman correlation coefficients are shown.

Visit	Site Group (N)	Initial Coccoid Cells (%) Mean±SE	Correlation Coefficient Pearson	Spearman	Initial Other (%) Mean±SE	Correlation Coefficient Pearson	Spearman
1-2	Loser	52.5±5.2	0.231	0.070	37.5±3.7	-0.180	-0.114
	Stable	56.5±1.5	0.164	0.028	31.9±1.0	0.060	0.063
	Improved	58.3±9.7	0.631	0.680	36.4±6.8	-0.741	-0.661
2-3	Loser	57.8±7.2	-0.107	-0.255	32.4±5.2	-0.089	0.164
	Stable	54.8±1.6	-0.016	-0.014	32.4±1.0	0.040	0.039
	Improved	44.0±8.9	-0.211	-0.138	39.6±6.5	-0.064	-0.037
3-4	Loser	53.2±6.1	-0.378	-0.383	39.6±4.5	0.416	0.382
	Stable	56.4±1.4	-0.022	0.018	34.0±1.0	-0.039	-0.029
	Improved	42.5±9.9	-0.008	0.000	50.2±7.3	0.066	0.000
4-5	Loser	46.7±8.4	0.350	0.336	42.9±6.1	-0.429	-0.450
	Stable	52.8±1.5	-0.020	-0.034	34.0±1.2	0.132	0.130
	Improved	59.9±8.7	-0.180	-0.347	32.3±6.5	0.264	0.426
5-6	Loser	53.9±5.8	0.216	0.187	28.1±4.1	-0.211	-0.058
	Stable	53.3±1.6	-0.009	-0.012	33.0±1.2	0.047	0.043
	Improved	40.7±11.3	-0.481	-0.516	40.8±8.2	0.115	-0.036
1-6	Loser	52.5±3.0	-0.138	-0.098	36.9±2.1	0.117	0.062
	Stable	57.5±1.6	-0.001	-0.015	31.3±1.1	0.080	0.098
	Improved	39.3±17.9	0.000	0.000	50.2±12.6	0.000	0.000

TABLE 4.19: Regression analysis and Spearman correlation test for the association of attachment level change over each two successive visits (and over one year) with motile rods and spirochaetes at the initial visit for each site group. Pearson and Spearman correlation coefficients are shown.

Visit	Site Group	(N)	Initial Motile Rods (%) Mean±SE	Correlation Coefficient Pearson	Correlation Coefficient Spearman	Initial Spirochaetes (%) Mean±SE	Correlation Coefficient Pearson	Correlation Coefficient Spearman
1-2	Loser		4.61±1.21	0.072	-0.026	5.35±2.05	-0.464*	0.075
	Stable		2.39±0.35	-0.037	-0.002	5.62±0.59	-0.031	0.047
	Improved		0.81±2.23	-0.337	-0.314	4.48±3.80	0.257	-0.160
2-3	Loser		1.99±1.53	0.257	0.411	7.31±2.42	0.223	0.088
	Stable		2.36±0.33	0.099	0.082	5.51±0.52	0.129	0.166
	Improved		7.41±1.96	0.419	0.175	8.96±3.02	0.212	0.257
3-4	Loser		2.98±0.99	0.144	0.056	4,23±2.45	-0.009	0.040
	Stable		2.05±0.23	-0.016	-0.077	6.21±0.56	0.000	-0.004
	Improved		1.50±1.61	0.540	0.598	5.71±4.01	-0.318	-0.359
4-5	Loser		1.70±1.42	-0.164	-0.268	8.72±2.74	-0.018	0.022
	Stable		2.39±0.26	-0.005	0.093	5.30±0.51	-0.005	0.078
	Improved		1.74±1.50	0.356	0.200	6.03±2.88	-0.248	-0.093
5-6	Loser		2.45±0.96	-0.074	0.080	6.86±2.41	0.163	0.074
	Stable		2.17±0.28	0.011	0.027	6.24±0.69	0.001	0.003
	Improved		4.50±1.88	0.228	0.577	14.00±4.74	0.684	0.698
1-6	Loser		4.20±0.72	0.127	0.121	6.43±1.22	0.023	0.050
	Stable		2.71±0.39	-0.004	0.043	5.57±0.66	-0.003	0.026
	Improved		6.93±4.23	0.000	0.000	3.66±7.21	0.000	0.000

* = statistically significant at $P < 0.05$

4.3.4 Laboratory findings of GCF CRP (GCF CRP concentration, GCF CRP secretion)

4.3.4.1 Reporting of GCF CRP results

GCF CRP analysis was reported using the concentration (the amount of CRP in a given volume), and the total CRP in the sample (activity/sample). Sites with converted volumes of equal to or < 0 uL were not included in the calculation of concentration as this made evaluation of the concentration data difficult. All sites with a converted GCF volume equal to 0 were included for secretion calculation. All sites were included when GCF volumes were calculated, and GCF volumes which converted to < 0 were adjusted to 0 uL.

4.3.4.2 GCF CRP levels for all periodontal sites in the patient and control groups

The sampled gingival crevicular fluid for each periodontal site at each visit in each group was tested for CRP level using ELISA technique described earlier (3.3.1). The results of GCF CRP were reported as the amount of CRP (nanogramm) per unit of GCF volume (uL). This was considered the concentration of GCF CRP, and expressed as ng/ml for data normalisation and analysis. The results were also reported as the total amount of CRP (ng) in the sample of GCF (activity/sample) collected over one minute, and this was the secretion of GCF CRP (ng/minute). The mean

levels of GCF CRP both for concentration and secretion for all sites for each subject group at each visit was shown in Table 4.20. Both GCF CRP concentration and secretion were significantly higher for periodontal sites in the patient group than those for the control group at each visit. The range of concentration was 0.0-1227.8 ng/ml and 0.0-118.5 ng/ml for all sites in the patient and control groups respectively. The mean GCF CRP concentration per site in the control group was 6.24ng/ml, while for the patient group was 60.0ng/ml for all visits.

The GCF CRP secretion per site ranged from 0.0-1.89 ng/ minute and 0.0-233.7ng/minute in the control and patient groups respectively. The mean CRP secretion for all periodontal sites for all visits was 1.23ng/minute and 18.8ng/minute in control and patient groups respectively. The range of means of GCF CRP concentration for all the sites was 53.87-63.52 ng/ml and 4.06-10.5 ng/ml in the patient and control groups respectively. The range of means of GCF CRP secretion was 16.19-20.82 ng/minute and 0.92-1.92ng/ minute for the patient and control groups respectively.

TABLE 4.20: Mean and Standard Error (SE) for gingival crevicular fluid (GCF) volume, GCF, CRP concentration (ng/ml) and GCF secretion (ng/minute) for all the sites in the control and patient groups.

Visit	Subject Group	No. of Sites	GCF Volume (uL)	CRP Concentration (ng/ml)	CRP Secretion (ng/minute)
1	Control	150	0.093±0.008	4.45±1.27	0.92±0.26
	Patient	324	0.267±0.015	63.52±4.96	19.46±1.60
2	Control	150	0.071±0.007	4.06±1.28	0.95±0.29
	Patient	324	0.267±0.014	60.17±5.83	19.73±1.51
3	Control	150	0.067±0.006	4.54±1.49	0.90±0.30
	Patient	324	0.242±0.012	53.87±3.92	16.19±1.32
4	Control	150	0.070±0.005	6.27±1.65	1.09±0.28
	Patient	324	0.287±0.014	59.97±4.20	19.06±1.39
5	Control	150	0.089±0.006	10.5±2.15	1.91±0.40
	Patient	312	0.275±0.016	62.75±4.33	17.66±1.14
6	Control	150	0.099±0.006	7.62±1.68	1.60±0.36
	Patient	312	0.312±0.016	59.17±3.48	20.82±1.50
All Visits	Control	900	0.081±0.003	6.24±0.66	1.23±0.13
	Patient	1920	0.279±0.006	59.90±1.85	18.81±0.58
Range:	Control		0.000-0.300	0.00-118.50	0.00-1.89
	Patient		0.000-1.461	0.00-1227.80	0.00-233.70

4.3.4.3 GCF CRP levels for each group of sites in periodontal patients during a longitudinal study

In this study, the periodontal sites for all patients were classified into three groups in the same way as mentioned before (2.7.2) using a safety threshold of 2mm. The levels of GCF CRP for both concentration and secretion are shown in Table 4.21. The results show the mean and standard error (SE) of GCF CRP levels for each site group, and these are CRP levels at the initial visit of each pair of successive visits for each site group. The loser sites were those sites which lost attachment level at the subsequent visit of each pair of successive visit, and each had GCF CRP levels which were measured at the initial visit of the same pair of successive visits. For visit 1-6, the loser sites were those sites which lost attachment level over one year, and their initial GCF CRP measurement was that found at the first visit corresponded to those sites designated as loser sites over the one year period. The same measurements were applied to both stable and improved sites for their initial GCF CRP levels.

As shown in Table 4.21, the GCF CRP levels were close for both loser and stable sites at the initial visits on most occasions. The mean GCF CRP concentration ranged from 26.5 to 68.4ng/ml, and from 52.6 to 64.0ng/ml in the loser and stable sites respectively. For the whole study (over one year), GCF CRP concentration was 57.2ng/ml and 65.5ng /ml

for loser and stable sites respectively.

For GCF CRP secretion which was reported as ng/minute (ng/sample), the range was 15.5-25.4 and 16.6-19.4ng/minute for loser and stable sites respectively. For the whole study, the GCF CRP secretion was 20.8 and 19.3 ng/minute for loser and stable sites respectively.

Comparisons in GCF CRP levels between loser and stable sites at the initial visit of each pair of successive visits were done and are shown later on in subsequent results.

The level of GCF CRP at the initial visit was considered as the initial GCF CRP level for each pair of successive visits in the following studies of its association with periodontal disease activity and in particular the attachment level loss which occurred at the subsequent visit.

TABLE 4.21: Mean and Standard Error (SE) of the gingival crevicular fluid (GCF) CRP concentration (ng/ml) and secretion (ng/minute) for each site group at the initial visit of each pair of successive visits (and for the period of one year).

			'GCF' CRP Levels at Initial Visit	
Visit	Site Group	Number	CRP Concentration (ng/ml) Mean±SE	CRP Secretion (ng/minute) Mean±SE
1-2	Loser	24	60.1±18.3	18.1±5.9
	Stable	293	64.0±5.2	19.4±1.7
	Improved	7	55.0±33.8	28.2±10.9
2-3	Loser	14	26.5±28.6	15.5±7.2
	Stable	301	61.2±6.1	19.0±1.6
	Improved	9	76.3±35.0	51.4±8.9
3-4	Loser	16	62.5±17.6	16.6±5.9
	Stable	302	52.6±4.1	16.0±1.4
	Improved	6	95.4±28.7	26.3±9.7
4-5	Loser	10	68.4±24.2	25.4±8.0
	Stable	293	59.7±4.4	19.1±1.5
	Improved	9	85.0±25.5	24.8±8.4
5-6	Loser	23	46.9±15.7	16.9±4.2
	Stable	283	62.3±4.5	17.5±1.2
	Improved	6	14.5±30.9	27.9±8.2
1-6	Loser	70	57.2±10.8	20.8±3.5
	Stable	240	65.5±5.8	19.3±1.9
	Improved	2	135.4±63.7	62.3±20.5

4.4 Association of GCF CRP levels with microbiologic findings, clinical parameters and GCF volume at each site group

4.4.1 Association of GCF CRP levels with subgingival bacterial morphotypes

In this study the GCF CRP levels (concentration and secretion) were tested for their association with subgingival bacteria including motile rods and spirochaetes. This study was carried out for each site group and for all sites collectively. Means of GCF CRP levels and mean percentages of motile rods and spirochaetes for each site group (and all sites together) at the initial visit of each pair of successive visits and of the whole period, were ranked. Spearman rank correlation test was used to test the correlation of GCF CRP levels with motile rods and spirochaetes. The results including Spearman correlation coefficients are shown in Table 4.22.

GCF CRP secretion had a significant positive correlation ($P<0.05$) with the percentage of motiles rods and spirochaetes for all sites together at the initial visit of most occasions, while it had a significant correlation with motile rods for the stable site group on most occasions and with spirochaetes on all occasions. CRP secretion had also a significant correlation with these bacteria for loser site group at the initial visit of the successive visits 4-5, 5-6 and for the one year period.

With regard to GCF CRP concentration, a significant positive correlation with motile rods,and with spirochaetes was found for each of loser and stable or site group as well as for all sites together on few occasions only.
For improved sites no significant correlation was found between GCF CRP levels and either of motile rods or spirochaetes on any occasion.

TABLE 4.22: Spearman rank correlation test between gingival crevicular fluid CRP levels and motile rods and spirochaetes for each site group in the periodontal patients at the initial visit of each pair of successive visits.

		Spearman Rank Correlation Coefficient			
		CRP Secretion and:		CRP Concentration and:	
Visit	Site Group	Motile rods (r)	Spirochaetes (r)	Motile rods (r)	Spirochaetes (r)
1-2	Loser(24)	0.054	-0.032	-0.194	-0.315
	Stable(293)	0.191	0.251*	0.106	0.140
	Improved(7)	0.195	0.341	0.195	0.341
	All(324)	0.171	0.244*	0.081	0.175
2-3	Loser(14)	0.147	0.259	0.463	0.490
	Stable(301)	0.169	0.270*	0.107	0.160
	Improved(9)	0.643	0.733	0.281	0.574
	All(324)	0.229*	0.293*	0.115	0.163
3-4	Loser(16)	0.061	0.013	0.202	-0.120
	Stable(302)	0.305*	0.246*	0.231*	0.262*
	Improved(6)	-0.392	0.007	-0.641	-0.158
	All(324)	0.336*	0.237*	0.224*	0.247*
4-5	Loser(10)	0.698*	0.671*	0.423	0.359
	Stable(293)	0.246*	0.216*	0.119	0.143
	Improved(9)	0.283	0.700	0.306	0.010
	All(312)	0.251*	0.248*	0.126	0.159
5-6	Loser	0.611*	0.810*	-0.783*	0.590*
	Stable(283)	0.205*	0.257*	0.131	0.266*
	Improved(6)	-0.157	0.750	-0.317	0.080
	All(312)	0.234*	0.315*	0.169	0.294*
1-6	Loser(70)	0.625*	0.260*	0.110	-0.137
	Stable(240)	0.239*	0.288*	0.262*	0.710*
	Improved(2)	None	None	None	None
	All(312)	0.297*	0.305*	0.221*	0.137

* = statistically significant at $P<0.05$

4.4.2 Association of GCF CRP levels with PI, GI, BOP

The purpose of this study was to find if GCF CRP levels were associated with the indices of inflammation, GI and BOP, and also with plaque index.

The association of GCF CRP levels including concentration and secretion with each of those indices for each site group and all sites together at the initial visit of each pair of successive visits and of the whole study over one year were tested using Fisher-test. The results were shown in Table 4.23. It was found that both GCF CRP secretion and concentration were significantly ($F< 0.05$) associated with plaque index, gingival index and bleeding on probing for the stable site group and for all sites together on most occasions.

For the loser site group, no significant correlation was found for CRP concentration with PI and GI or BOP, except on one occasion for GI (visits 1-6), and on one occasion for BOP (visits 5-6).

With regard to GCF CRP secretion in the loser sites, a significant positive correlation ($F< 0.05$) was found for visits 3-4 and 1-6 for PI, for visits 5-6 and 1-6 for GI, while for BOP on most occasions.

No significant correlation was found between CRP concentration and secretion with PI, GI and BOP for the improved site group on any occasion.

TABLE 4.23: Fisher test analysis for the association of gingival crevicular fluid CRP levels with plaque index (PI), gingival index (GI) and bleeding on probing (BOP) for each site group at the initial visit of each pair of successive visits.

		Fisher test analysis and F-value					
		CRP concentration and:			CRP secretion and:		
Visit	Site Group	PI F-Value	GI F-Value	BOP F-Value	PI F-Value	GI F-Value	BOP F-Value
1-2	Loser(24)	0.111	0.636	0.866	0.476	0.964	0.016
	Stable(293)	0.012	<0.001	<0.001	<0.001	<0.001	<0.001
	Improved(7)	0.371	None	0.120	0.642	None	0.111
	All(324)	0.017	<0.001	<0.001	<0.001	<0.001	<0.001
2-3	Loser(14)	0.438	0.637	0.089	0.726	0.656	0.023
	Stable(301)	0.019	<0.001	<0.001	<0.001	<0.001	<0.001
	Improved(9)	0.424	0.515	0.424	0.049	0.099	0.049
	All(324)	0.014	<0.001	<0.001	<0.001	<0.001	<0.001
3-4	Loser(16)	0.109	0.520	0.104	0.049	0.980	0.199
	Stable(302)	0.002	0.016	<0.001	<0.001	<0.001	<0.001
	Improved(6)	0.270	0.280	0.804	0.361	0.201	0.427
	All(324)	<0.001	0.014	<0.001	<0.000	<0.001	<0.001
4-5	Loser(10)	0.138	0.396	0.056	0.080	0.715	0.046
	Stable(293)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Improved(9)	0.391	None	0.644	0.420	None	0.145
	All(312)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
5-6	Loser(23)	0.660	0.628	0.029	0.153	0.003	<0.001
	Stable(283)	<0.001	0.213	<0.001	<0.001	<0.001	<0.001
	Improved(6)	0.923	0.490	0.313	0.951	0.550	0.950
	All(312)	<0.001	0.259	<0.001	<0.001	<0.001	<0.001
1-6	Loser(70)	0.588	0.046	0.581	0.044	<0.001	0.023
	Stable(240)	<0.001	0.028	<0.001	<0.001	<0.001	<0.001
	Improved(2)	None	None	None	None	None	None
	All(312)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

4.4.3 Association of GCF CRP levels with PD and GCF volume

In this study, GCF CRP levels were tested for their association with pocket depth and gingival crevicular fluid volume for all sites and for each of the site groups which are loser, stable and improved sites.

The GCF volume and pocket depth for all sites and for each site group at the initial visit of each pair of successive visits and for the period of one year, was ranked. The GCF CRP level for each concentration and secretion at the same initial visit was also ranked. Spearman rank correlation was used to test the association of GCF CRP with each of these two variables. The results were shown in Table 4.24.

For pocket depth, it was found that GCF CRP levels for both secretion and concentration showed a positive significant correlation ($P<0.05$) with pocket depth for loser site group, stable site group and all sites together on most occasions.

For GCF volume, a significant positive correlation was found with CRP concentration on one occasion only for each of loser and stable site group and for all the sites together. Interestingly GCF CRP secretion was significantly associated with GCF volume on all occasions for each site group and for the total sites together. On other hand, no correlation was found for GCF CRP concentration for each of pocket depth and GCF volume for improved sites on any occasion.

It was obvious that GCF CRP levels (secretion and concentration) were significantly associated with both pocket depth and GCF volume for loser, stable and all sites together at the initial visit of the successive visits 1-6 (over one year).

TABLE 4.24: Spearman correlation test between gingival crevicular fluid (GCF) CRP levels, pocket depth and GCF volume for each site group in periodontal patients at the initial visit of each pair of successive visits.

			Spearman Correlation Coefficient Between			
			CRP concentration and:		CRP secretion and:	
Visit	Site Group	Number	Pocket Depth (r)	GCF Volume (r)	Pocket Depth (r)	GCF Volume (r)
1-2	Loser	24	0.392*	-0.226	0.279	0.472*
	Stable	293	0.247*	0.169	0.267*	0.600*
	Improved	7	0.699	0.431	0.272	0.881*
	All	324	0.247*	0.141	0.266*	0.584*
2-3	Loser	14	0.741*	0.428	0.655*	0.780*
	Stable	301	0.267*	0.156	0.384*	0.623*
	Improved	9	0.530	0.379	0.348	0.824*
	All	324	0.149	0.267*	-0.235*	0.644*
3-4	Loser	16	-0.110	0.953	0.060	0.483*
	Stable	302	0.300*	0.243*	0.332*	0.650*
	Improved	6	-0.651	-0.162	0.626	0.993*
	All	324	0.291*	0.237*	0.334*	0.652*
4-5	Loser	10	0.659*	0.174	0.711*	0.901*
	Stable	293	0.228*	0.143	0.357*	0.619*
	Improved	9	0.065	0.079	0.414	0.777
	All	312	0.253*	0.150	0.374*	0.639*
5-6	Loser	23	0.233	0.041	0.414*	0.486*
	Stable	283	0.333*	0.087	0.434*	0.607*
	Improved	6	-0.449	-0.562	0.383	0.532
	All	312	0.321*	0.060	0.432*	0.586*
1-6	Loser	70	-0.391*	-0.415*	-0.343*	0.469*
	Stable	240	0.368*	0.257*	0.417*	0.682*
	Improved	2	None	None	None	None
	All	312	0.323*	0.175	0.471*	0.643*

* = statistically significant at $P<0.05$

4.4.4 Association of GCF CRP levels with attachment level

4.4.4.1 Association of the change in GCF CRP level with attachment level change for all sites in the control and patient groups

The purpose of this study was to find if the response in GCF CRP levels was associated with attachment level change over each paired successive visits and over one year for all sites in both patient and control groups. It was also the aim to find if there was any difference between the control and patient groups for this response.

The change in attachment level for all the periodontal sites in each group over each prescribed period, and the change in GCF CRP levels for secretion and the logarithmic transformation for CRP concentration change for the same sites were subjected to regression analysis and Pearson correlation coefficient was produced. The results of this analysis and Pearson correlation coefficients are shown in Table 4.25.

As can be seen from the results, a significant positive correlation ($P<0.05$) was found between attachment level change over each pair of successive visits and over one year with the change in GCF CRP levels both for secretion and concentration for all the sites in the patient group. The correlations are illustrated in figures 4.4-9,and it was found that as the probing attachment level increased

between each two successive visits (and over one year), the GCF CRP levels increased over the same period. This means that GCF CRP was present in high levels when there was increasing probing attachment level at subsequent visit of each pair of successive visits and after one year. It was also found that when the probing attachment level decreased, the GCF CRP level decreased over the same period. So high levels of GCF CRP were associated with increasing probing attachment level, while low levels of GCF CRP were associated with decreasing probing attachment levels.

A significant correlation was also found between attachment level change and the change in GCF CRP levels for periodontal sites in the patient group for all visits together (Table 4.25, and Figures 4.8-9).

For the control group, no significant correlation was found between attachment level change and the change in GCF CRP levels for either secretion or concentration for all sites on any occasion.

The results of above studies led to further investigation of GCF CRP levels and clinical parameters used in measuring periodontal breakdown and in particular the attachment level loss.

TABLE 4.25: Regression analysis and Pearson correlation coefficient (r) for the change in attachment level and gingival crevicular fluid (GCF) CRP levels (concentration and secretion) between each pair of successive visits, and over one year, for all periodontal sites in the patient and control groups.

			Pearson Correlation Coefficient (r) between Attachment Level Change and CRP Level Change	
Visit	Group	Sites	(r) value	(r) value
1-2	Control	150	-0.072	-0.089
	Patient	324	0.307*	0.275*
2-3	Control	150	0.108	0.071
	Patient	324	0.212*	0.289*
3-4	Control	150	0.117	0.093
	Patient	324	0.236*	0.266*
4-5	Control	150	0.048	0.102
	Patient	312	0.207*	0.208*
5-6	Control	150	-0.031	0.070
	Patient	312	0.333*	0.331*
1-6	Control	150	0.062	0.080
	Patient	312	0.313*	0.288*
All Visits	Control	750	0.073	0.083
	Patient	1546	0.300*	0.300*

* = statistically significant at $P<0.05$

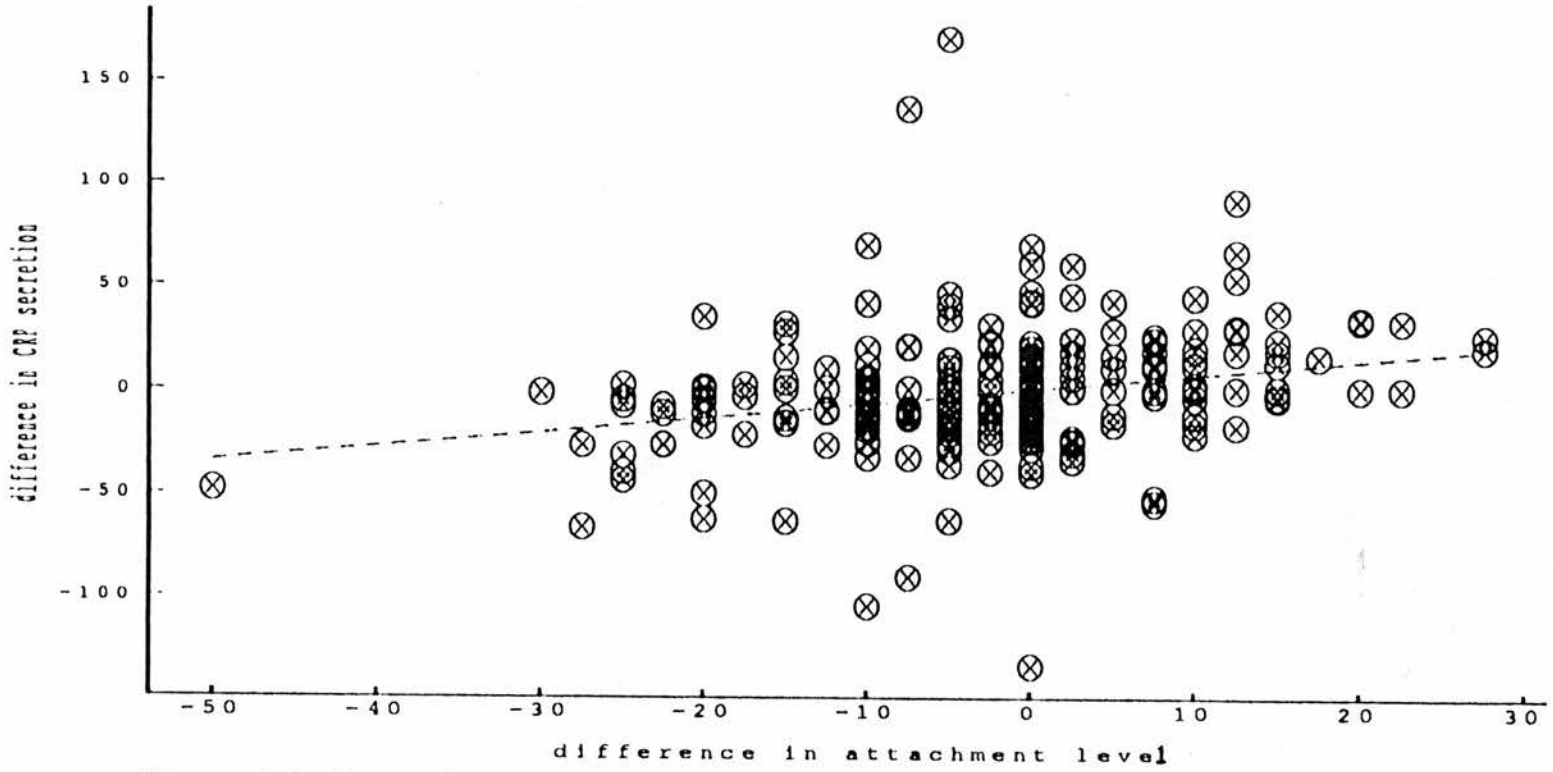
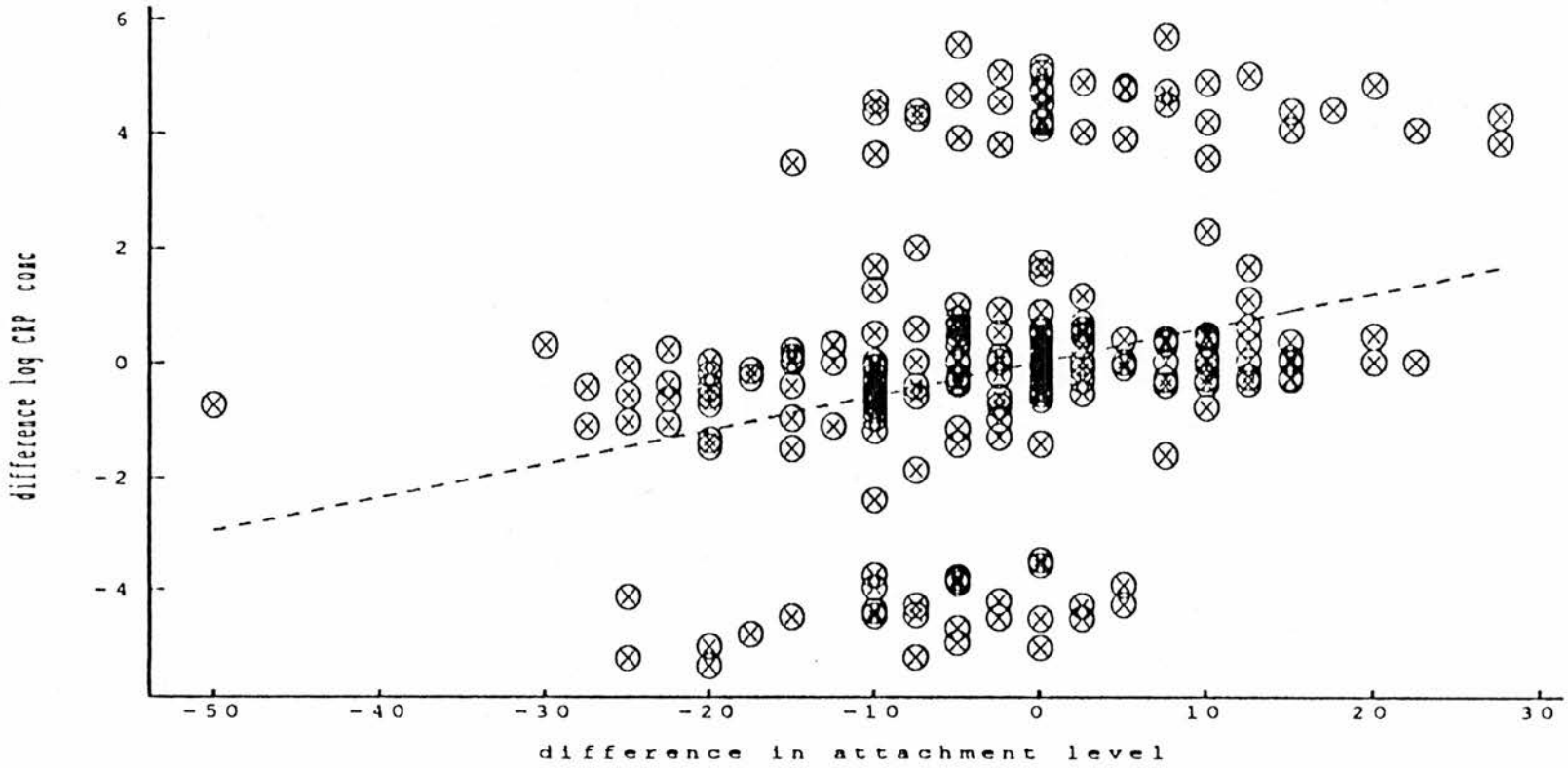


Figure 4.4 Regression analysis of the differences in the attachment level (mm) and CRP secretion (ng/minute) for all the sites in the patient group for the succesive visits 1-2. Pearson correaltion coefficient is statistically significant (r=0.275 at significance level less than 0.05).



4.5 Regression analysis of the differences in the attachment level (mm) and logarithmic CRP concentration (ng/ml) for all the sites in the patient group for the successive visits 1-2. Pearson correlation coefficient is statistically significant (r=0.307 at significance level less than 0.05).
Conc. = concentration
log. = logarithm

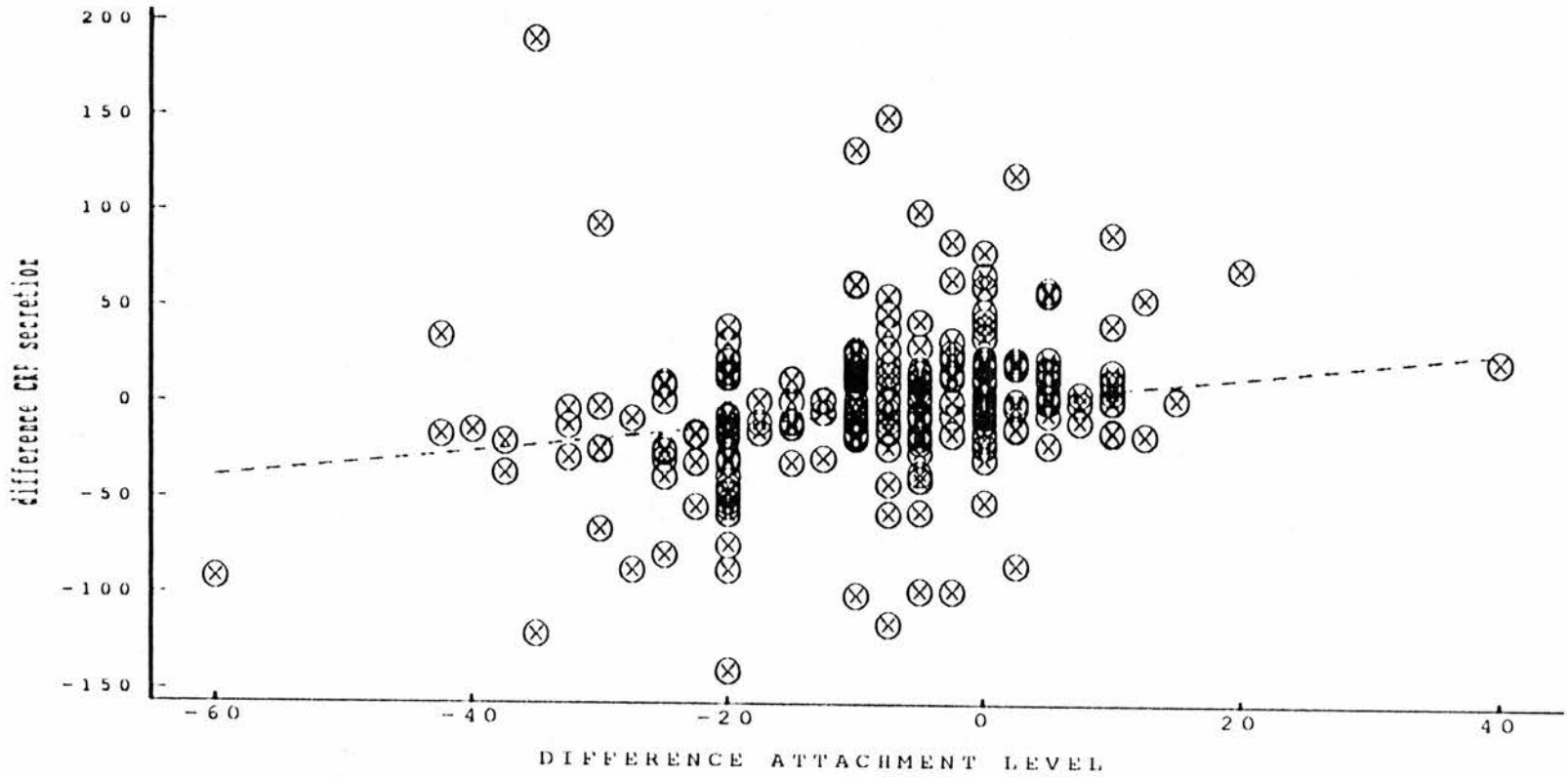


Figure 4.6 Regression analysis of the differences in the attachment level (mm) and CRP secretion (ng/minute) for all the sites in the patient group over the one year period (visits 1-6). Pearson correlation coefficient is statistically significant (r=0.288 at significance level less than 0.05).

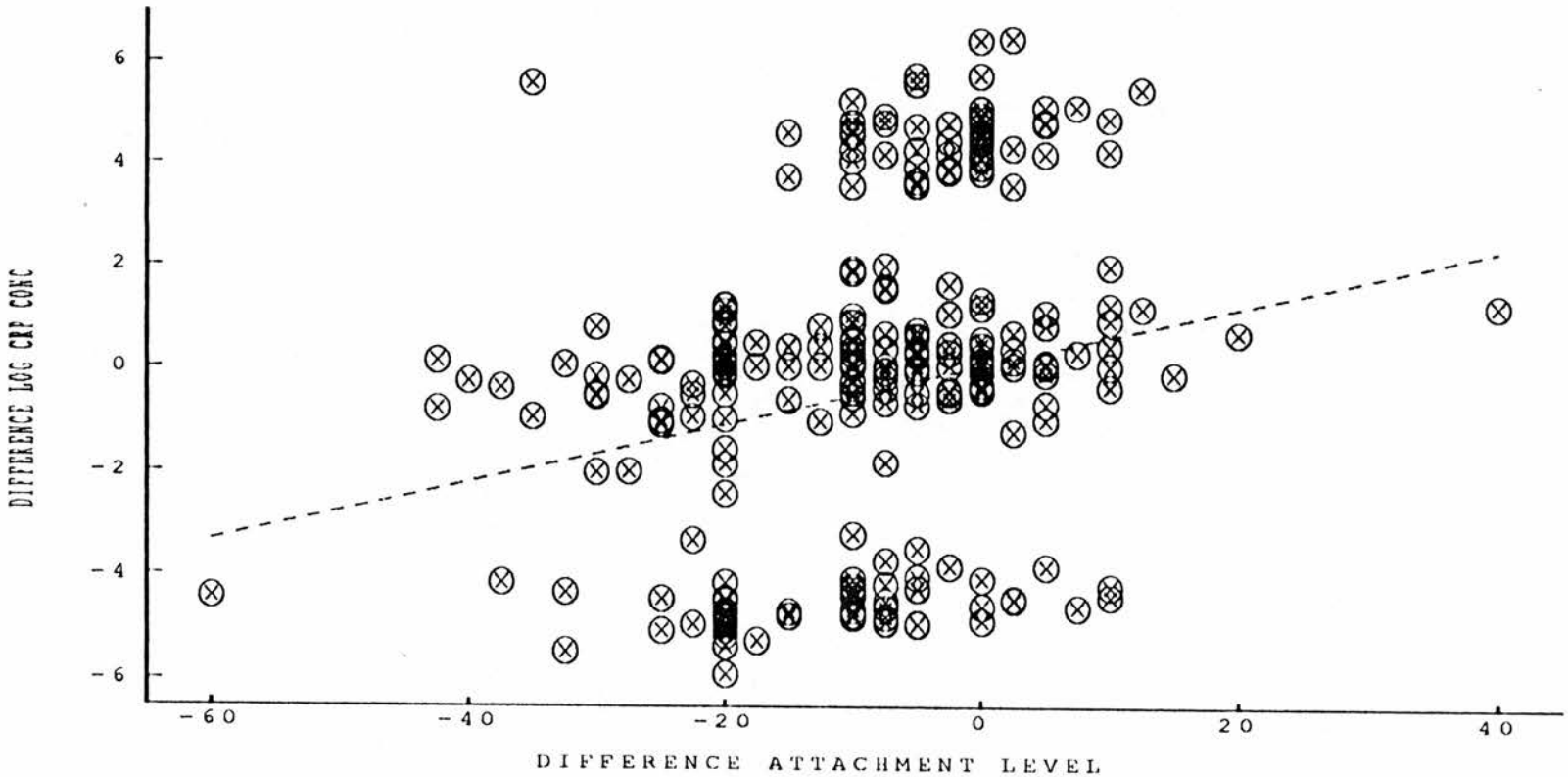
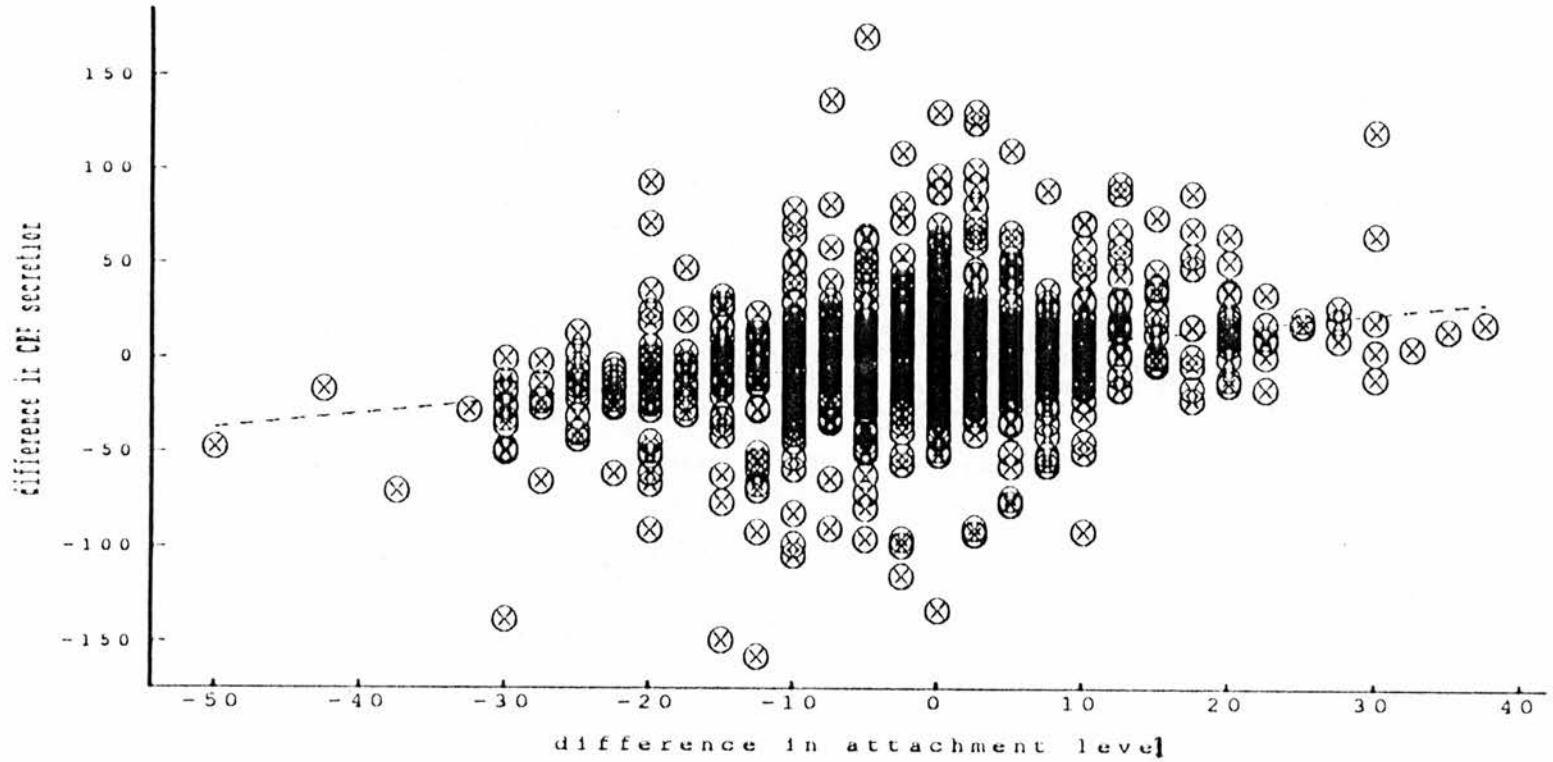
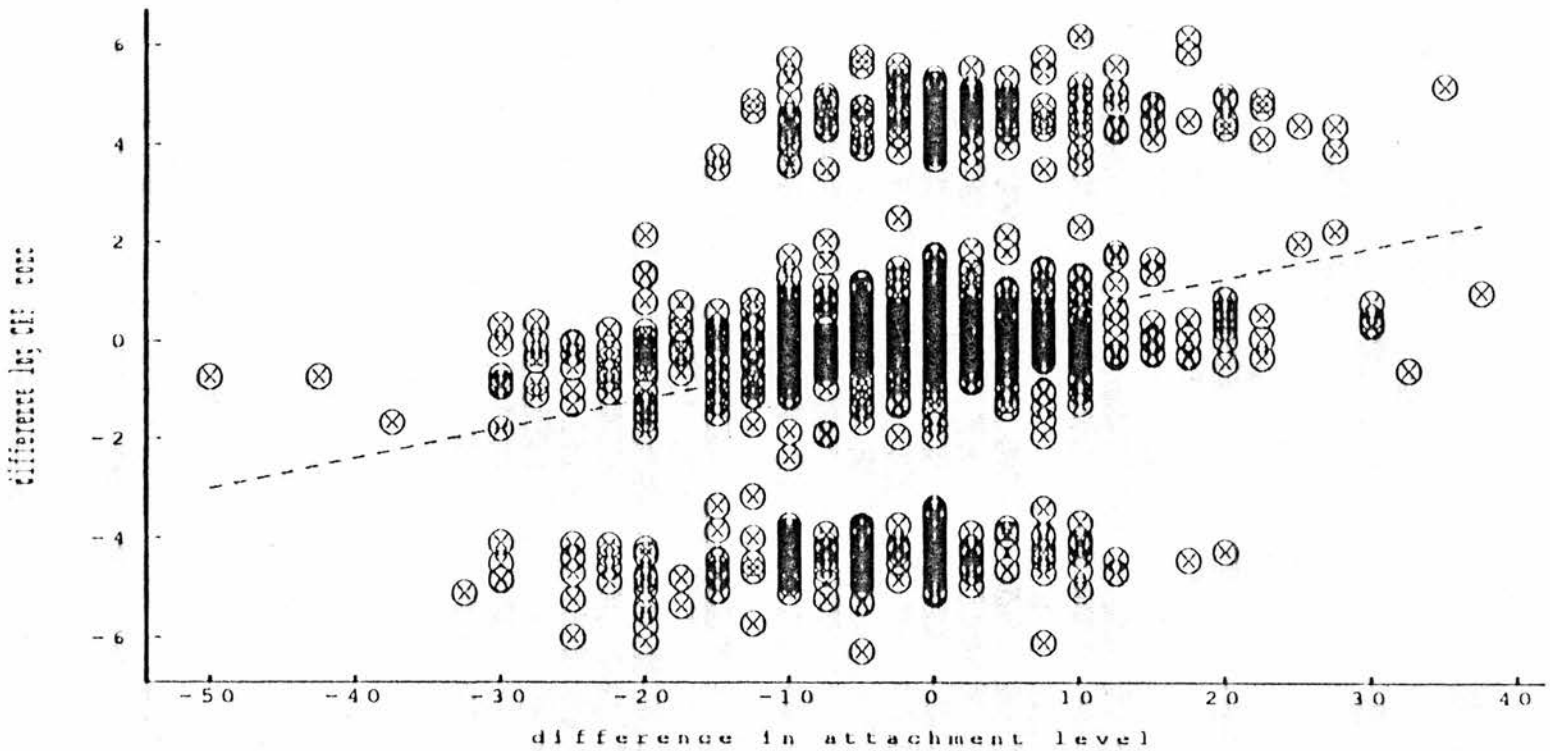


Figure 4.7 Regression analysis of the differences in the attachment level (mm) and logarithmic CRP concentration (ng/ml) for all the sites in the patient group over the one year period (visits 1-6). Pearson correlation coefficient is statistically significant (r=0.313 at significance level less than 0.05).
Conc.=concentration
log.=logarithm



4.8 Regression analysis of the differences in the attachment level and CRP secretion (ng/minute) for all the sites in the patient group over all the visits. Pearson correlation coefficient is statistically significant (r=0.300 at significance level less than 0.05).



4.9 Regression analysis of the differences in the attachment level and logarithmic CRP concentration (ng/ml) for all the sites in the patient group over all the visits. Pearson correlation coefficient is statistically significant (r=0.300 at significance level less than 0.05).
Conc.=concentration
log.=logarithm

4.4.4.2 Association of GCF CRP levels with attachment level change at each site group in periodontal patients

The purpose of this study was to find if GCF CRP levels, for both secretion and concentration, were associated with or could predict periodontal disease activity measured by attachment level loss. The three groups of sites; loser, stable and improved sites were used for this study. The attachment level change per each site group over each two successive visits, and over one year and GCF CRP levels (concentration and secretion) measured at the initial visit of each pair of successive visits, and at the initial visit related to attachment level change over one year, were all subjected to regression analysis. The results of this analysis using Pearson correlation coefficient and Spearman rank correlation coefficient for both CRP secretion and concentration with attachment level change, are shown in Table 4.26.

It was obvious that no significant correlation was found between GCF CRP levels, for both concentration and secretion, with attachment level change for any site group on any occasion.

TABLE 4.26: Regression analysis using Pearson correlation coefficient (r1) and Spearman rank correlation coefficient (r2) for the association of attachment level change for each site group over each pair of successive visits (and over one year) with gingival crevicular fluid (GCF) CRP measured at the initial visit of each pair of sucessive visits in the patient group.

		Correlation test between attachment level change and GCF CRP levels							
		Concentration		Secretion		Concentration		Secretion	
Visit	Site Group	r1	P	r2	P	r1	P	r2	P
1-2	Loser (24)	0.029	NS	0.077	NS	-0.038	NS	0.046	NS
	Stable(293)	0.143	NS	0.033	NS	0.114	NS	0.071	NS
	Improved(7)	-0.114	NS	-0.251	NS	-0.238	NS	-0.315	NS
2-3	Loser(14)	-0.060	NS	0.120	NS	0.136	NS	0.097	NS
	Stable(301)	0.078	NS	0.040	NS	0.050	NS	0.028	NS
	Improved(9)	0.563	NS	0.296	NS	0.516	NS	0.230	NS
3-4	Loser(16)	0.238	NS	0.372	NS	0.216	NS	0.332	NS
	Stable(302)	0.082	NS	0.048	NS	0.013	NS	0.008	NS
	Improved(6)	-0.443	NS	-0.527	NS	-0.239	NS	-0.598	NS
4-5	Loser(10)	0.133	NS	0.254	NS	-0.098	NS	-0.118	NS
	Stable(293)	0.082	NS	0.179	NS	0.089	NS	-0.144	NS
	Improved(9)	0.680	NS	0.097	NS	0.694	NS	0.073	NS
5-6	Loser(23)	0.223	NS	0.076	NS	0.194	NS	0.042	NS
	Stable(283)	0.007	NS	-0.042	NS	-0.076	NS	-0.092	NS
	Improved(6)	-0.033	NS	0.853	NS	0.030	NS	0.698	NS
1-6	Loser(70)	0.015	NS	-0.156	NS	-0.061	NS	-0.106	NS
	Stable(240)	0.135	NS	0.078	NS	0.118	NS	0.112	NS
	Improved(2)	0.000	NS	0.000					

NS = not significant at $P<0.05$

4.5 Differences in clinical parameters, subgingival bacteria and GCF CRP levels among site groups

The purpose of this study was to investigate the differences between site groups (loser versus stable and loser versus improved) for their clinical parameters (PI, GI, BOP and PD), GCF volume, GCF CRP concentration and secretion, and subgingival bacteria (coccoid cells,other, motile rods and spirochaetes) at the initial visit of each pair of successive visits and the initial visit for one year period. Fisher-test was used to test the differences among all site groups together for all these variables except for GI,PI and BOP. When F-value was significant, analysis of variance (t-test) was used to test the difference between each two group of sites.

For PI, GI and BOP, Chi-square was applied to test the difference between loser versus stable sites for these clinical indices.

The results for these analyses are shown in Tables 4.27-4.29.

For both CRP secretion and concentration (Table 4.27), no significant differences was found between loser and stable sites, and between loser and improved sites at the initial visit on almost all occasions.

For GCF volume (Table 4.27), a significant difference ($F<0.05$) was found among site groups for visits 1-2, 2-3 and 1-6. ANOVA was used to test the difference between each two site groups, and it was found that GCF volume was

significantly ($P<0.05$) higher in loser sites than for stable sites.

For pocket depth (Table 4.27), a significant difference ($F<0.05$) was found among the site groups together on all occasions. ANOVA again was used to test the difference between loser and stable sites and between loser and improved sites. No significant difference was found between loser and stable sites on all occasions.

For PI and GI (Table 4.28), no significant difference was found between loser and stable sites on any occasion.

For BOP, the frequency of bleeding sites was significantly higher for loser site group than for stable site group (X_2 value =39.9) only for visits 1-6.

For subgingival bacteria (Table 4.29), no significant difference was found among all site groups for all bacterial morphotypes (coccoid cells, other, motile rods and spirochaetes) on any occasion.

TABLE 4.27: F-test for the differences between all site groups for their gingival crevicular fluid (GCF) CRP concentration, GCF CRP secretion, GCF volume and pocket depth (PD) recorded at the initial visit of each pair of successive visits. When the F-value was significant, ANOVA was used to test the difference in that variable between loser and stable sites, and between loser and improved sites.

		F-test	Analysis of Variance (ANOVA)	
Visit	Variable	All Site Groups F-Value	Loser Versus Stable T-Value	Loser Versus Improved T-Value
1-2	CRP Concentration	0.947	-	-
	CRP Secretion	0.705	-	-
	GCF Volume	0.018	2.35*	-13.09*
	Pocket Depth	<0.007	1.84	4.6*
2-3	CRP Concentration	0.432	-	-
	CRP Secretion	0.002	0.62	3.15*
	GCF Volume	<0.001	2.71*	-2.26*
	Pocket Depth	<0.001	1.49	-9.23*
3-4	CRP Concentration	0.297	-	-
	CRP Secretion	0.570	-	-
	GCF Volume	0.768	-	-
	Pocket Depth	<0.001	0.17	-8.81*
4-5	CRP Concentration	0.589	-	-
	CRP Secretion	0.604	-	-
	GCF Volume	0.923	-	-
	Pocket Depth	0.003	1.03	-7.80*
5-6	CRP Concentration	0.018	1.02	2.88*
	CRP Secretion	0.446	-	-
	GCF Volume	0.465	-	-
	Pocket Depth	<0.001	-0.26	-9.90*
1-6	CRP Concentration	0.424	-	-
	CRP Secretion	0.105	-	-
	GCF Volume	0.006	2.56*	18.30*
	Pocket Depth	0.004	-1.20	-1.92

* = statistically significant at $P<0.05$ for t-value

STABLE4.28: Chi-square analysis of the difference between loser and stable sites for their plaque index (PI), gingival index (GI) and bleeding on probing (BOP) recorded at the initial visit of each pair of successive visits and the baseline visit for a one-year period. X^2 values are shown.

		Chi-square Analysis Loser Sites Versus Stable Sites
Visit	Clinical Index	X^2 Value
1-2	PI	1.26
	GI	0.04
	BOP	0.83
2-3	PI	0.24
	GI	0.10
	BOP	0.05
3-4	PI	0.25
	GI	0.22
	BOP	0.30
4-5	PI	0.18
	GI	0.14
	BOP	0.29
5-6	PI	4.90
	GI	0.02
	BOP	0.97
1-6	PI	4.50
	GI	4.52
	BOP	39.90*

* = statistically significant at $P<0.05$

TABLE 4.29: F-test for the differences between all site groups for their percentages of coccoid cells, other motile rods and spirochaetes recorded at the initial visit of each pair of successive visits. When the F-value was significance, ANOVA was used to test the differences in that variable between loser and stable sites, and between loser and improved sites.

		F- test	Analysis of Variance (ANOVA)	
		All Site Groups	Loser Versus Stable	Loser Versus Improved
Visit	Variable	F-value	T-Value	T-Value
1-2	Coccoid Cells	0.748	-	-
	Other	0.294	-	-
	Motile Rods	0.244	-	-
	Spirochaetes	0.951	-	-
2-3	Coccoid Cells	0.453	-	-
	Others	0.551	-	-
	Motile Rods	0.033	0.10	0.30
	Spirochaetes	0.359	-	-
3-4	Coccoid Cells	0.349	-	-
	Others	0.055	-	-
	Motile Rods	0.613	-	-
	Spirochaetes	0.732	-	-
4-5	Coccoid Cells	0.546	-	-
	Others	0.347	-	-
	Motile Rods	0.820	-	-
	Spirochaetes	0.466	-	-
5-6	Coccoid Cells	0.339	-	-
	Others	0.327	-	-
	Motile Rods	0.461	-	-
	Spirochaetes	0.265	-	-
1-6	Coccoid Cells	0.217	-	-
	Others	0.056	-	-
	Motile Rods	0.123	-	-
	Spirochaetes	0.792	-	-

4.6 GCF CRP levels for loser and stable periodontal sites at the initial visit, at the subsequent visit of each pair of successive visits, and their differences over the visits

4.6.1 GCF CRP levels for all loser and all stable periodontal sites for all patients

The purpose of this study was to find the level of GCF CRP for concentration (ng/ml) and secretion (ng/minute or ng/sample) for all the sites in the loser group and for the stable group, at the initial visit and subsequent visit of each pair of successive visits. It was also the aim to find if there was a significant difference between loser versus stable sites at the initial and at the subsequent visit, and if there was a significant difference in their CRP level responses (change) over each two successive visits for 2-months interval and over one year. Furthermore, it was also aimed to study the difference in GCF CRP levels for each site group (loser or stable) between the initial and subsequent visits. In this study all the loser sites and all the stable sites had already been identified over each two successive visits using safety threshold of 2mm. The number and mean +_ standard error of each group of these sites for each pair of successive visits and over one year have already been shown in Table 4.2.

4.6.1.1 The levels of GCF CRP concentration and secretion

The level of GCF CRP for concentration and secretion for each site was determined using ELISA technique was described earlier (3.3.1). The mean GCF CRP concentration and secretion for all loser sites and for all stable sites at the initial visit, subsequent visit and their difference between these two visits are shown in Tables 4.30 and 4.31. As is shown in Table 4.30, the mean CRP concentration at the initial visit was ranged from 26.5 to 68.4 ng/ml and from 42.6 to 65.0 ng/ml for the loser and stable site respectively. The mean CRP concentration at the subsequent visit had a range of 109.3-177.9 ng/ml and of 42.8-61.1 ng/ml for the loser and stable sites respectively.

For CRP secretion, as is shown in Table 4.31, the mean CRP secretion at the initial visit had a range of 15.5-25.4 ng/ml and of 16.0-19.4 ng/ml, while the means at subsequent visit ranged 30.6-51.0 ng/ml and 14.4-19.7 ng/ml for loser and stable sites respectively.

TABLE 4.30: Gingival crevicular fluid CRP concentration (ng/ml) for loser and stable sites for the patient group at the initial visit, subsequent visit, and their difference for each pair of successive visits (and over one year).

			CRP Concentration (ng/ml)		
Visits	Site Group	Number	Initial Visit	Subsequent Visit	Difference
1-2	Loser	24	60.1	124.7	-64.6
	Stable	293	64.0	56.1	8.1
2-3	Loser	14	26.5	177.9	-151.3
	Stable	301	61.2	48.0	3.2
3-4	Loser	11	62.5	127.3	-64.7
	Stable	302	52.6	56.7	-4.3
4-5	Loser	10	68.4	128.1	-59.7
	Stable	293	59.7	61.2	-1.6
5-6	Loser	23	46.9	109.3	-62.3
	Stable	283	62.3	55.4	6.8
1-6	Loser	70	57.2	117.2	-59.3
	Stable	240	65.2	42.8	23.0

TABLE 4.31: Gingival crevicular flud CRP secretion (ng/minute) for loser and stable sites for the patient group at initial visit, subsequent visit, and their difference over each pair of successive visits (and over one year).

			Mean CRP Secretion (ng/minute)		
Visit	Site Group	Number	Initial Visit	Subsequent Visit	Difference
1-2	Loser	24	18.1	37.2	-19.1
	Stable	293	19.4	18.6	0.8
2-3	Loser	14	15.5	51.0	-35.5
	Stable	301	19.0	14.4	4.6
3-4	Loser	16	16.6	35.9	-19.3
	Stable	302	16.0	18.3	-2.3
4-5	Loser	10	25.4	30.6	-5.3
	Stable	293	19.1	17.3	1.7
5-6	Loser	23	16.9	37.4	-20.5
	Stable	283	17.5	19.7	-2.2
1-6	Loser	70	20.8	41.1	-20.5
	Stable	240	19.3	15.1	4.6

4.6.1.2 Comparison of GCF CRP levels at all loser and all stable sites

In this study, the levels for CRP secretion and concentration were compared between loser and stable sites (loser versus stable) at the initial visit, subsequent visit and the difference between these two visits. The concentration and secretion levels were also compared for the same site group (loser versus loser, and stable versus stable) between the initial and subsequent visits. ANOVA (t-test) was used to compare all these data, and the results are shown in Tables 4.32 and 4.33.

For CRP concentration, as is shown in Table 4.32, no significant difference was found between loser and stable sites (loser versus stable) for their CRP concentration at the initial visits for all occasions, while a highly significant ($P<0.05$) difference was found in their CRP concentrations at the subsequent visit for all occasions. The CRP concentration for all loser sites was significantly higher than that for all stable sites at the subsequent visit on all occasions.

It was also found that the response (change) of CRP concentration for each site group between these two visits (initial and subsequent) was significantly different ($P<0.05$) between loser and stable sites (loser versus stable) at all occasions, with significantly higher responses at loser sites than at stable sites. On the other hand, it was found that CRP concentration was significantly

higher ($P<0.05$) for loser sites at the subsequent visit than at initial visits (loser versus loser) on all occasions, while no significant difference was found in CRP concentration for stable sites between initial and subsequent visits (stable versus stable) on most occasions (except visits 2-3 and 1-6).

For CRP secretion, as is shown in Table 4.33, there was a significant ($P<0.05$) difference in its level between loser and stable sites (loser versus stable) at the subsequent visit but not at the initial visit. A significantly higher CRP secretion was found for loser sites than for stable sites at subsequent visits, and a significantly higher response in CRP secretion was found for loser sites compared to stable sites (loser versus stable) between these two visits (initial and subsequent). It was also found that there was a significantly higher level of CRP secretion for loser sites at the subsequent visit when compared with the level at the initial visit (loser versus loser between visits), while no significant difference in CRP secretion was found for stable versus stable site between these two visits except on one occasion.

TABLE 4.32: Analysis of variance (ANOVA) for gingival crevicular fluid CRP concentration between the loser and stable site groups, at the initial visit, subsequent visit, and between their differences over each pair of successive visits (and over one year). ANOVA for CRP concentration for each site group between each two successive visits is also shown.

			Analysis of Variance and T-Value				
			At the Same Visit		Between Two visits		
Successive Visits	Site Group	Number	Loser Versus Stable Initial Visit	Loser Versus Stable Subsequent Visit	Loser Versus Stable	Loser Versus Loser	Stable Versus Stable
1-2	Loser	24	0.20	3.106*	-3.00*	-2.77*	1.22
	Stable	293					
2-3	Loser	14	1.20	7.20*	-5.70*	-5.36*	2.17*
	Stable	301					
3-4	Loser	16	0.54	3.68*	-2.63*	-2.82*	-0.78
	Stable	302					
4-5	Loser	10	0.34	2.73*	-2.00*	-2.08*	-0.30
	Stable	293					
5-6	Loser	23	0.95	4.13*	-3.93*	-3.68*	1.41
	Stable	283					
1-6	Loser	70	0.67	10.28*	-5.79*	-4.75*	3.41*
	Stable	240					

* = statistically significant at $P<0.05$

TABLE 4.33: Analysis of variance (ANOVA) of gingival crevicular fluid CRP secretion between loser and stable sites in patient group at initial visit, subsequent visit, and between their difference between these successive visits (and over one year). ANOVA of the GCF CRP level difference for each site group (loser versus loser, stable versus stable) between each pair of successive visits is also shown.

			Analysis of Variance and T-Value				
			At the Same Visit		Between Initial and Subsequent Visits		
Successive Visits	Site Group	Number	Loser Versus Stable Initial Visit	Loser Versus Stable Subsequent Visit	Loser Versus Stable	Loser Versus Loser	Stable Versus Stable
1-2	Loser	24	0.20	3.23*	-3.62*	-3.62*	0.53
	Stable	293					
2-3	Loser	14	0.50	5.88*	-5.58*	-4.98*	3.00*
	Stable	301					
3-4	Loser	16	0.10	2.75*	-2.50*	-2.95*	-1.51
	Stable	302					
4-5	Loser	10	0.78	2.06*	-1.98*	-3.06*	1.25
	Stable	293					
5-6	Loser	23	0.14	3.17*	-3.38*	-3.96*	-1.36
	Stable	283					
1-6	Loser	70	0.34	7.95*	-5.309*	-2.71*	0.26
	Stable	240					

* = statistically significant at $P<0.05$

4.6.2 GCF CRP levels for matched pairs of the most loser site and the most stable periodontal site in the patient group

In this study, a matched pair of loser and stable sites within each patient was selected. This selection was based on the worst site (the most loser) and the most stable site. One loser site and one stable site within the loser patient were selected, and whenever there was more than one stable site, a random selection was used. The mean and standard deviation (SD) of the attachment level change for the matched pairs of the selected sites over each pair of successive visits (and for the one year period), are shown in Table 4.34.

4.6.2.1 The level of GCF CRP secretion and concentration

The levels of GCF CRP for concentration and secretion for matched pairs of sites, at the initial visit and subsequent visit of each pair of successive visits (and over one year),and their CRP level difference between these visits are shown in Table 4.35 and Table 4.36.

TABLE 4.34 Mean and Standard Deviation (SD) of attachment level change for matched pairs of loser and stable sites within each patient, over each pair of successive visits, and over one year.

Visit	Site Group	Number	Attachment Level Change Mean ± SD
1-2	Loser	17	-2.45±0.72
	Stable	17	-0.12±0.27
2-3	Loser	11	-2.28±0.38
	Stable	11	0.20±0.22
3-4	Loser	16	-2.38±0.40
	Stable	16	0.19±0.21
4-5	Loser	10	-2.63±0.69
	Stable	10	0.20±0.22
5-6	Loser	19	-2.41±0.54
	Stable	19	0.23±0.30
1-6	Loser	36	-2.73±0.93
	Stable	36	0.18±0.26

TABLE 4.35: Mean and Standard Deviation (SD) of gingival crevicular fluid CRP concentration (ng/ml) for matched pairs of loser and stable sites within each patient at the initial visit, subsequent visit and between each pair of these successive visits, and over one year.

Visits	Site Group	Number	CRP Concentration (ng/ml) Mean ± SD Initial Visit	Subsequent Visit	Difference
1-2	Loser	17	70.8±64.9	118.8±63.0	-48.9±66.0
	Stable	17	73.7±98.5	43.0±48.9	31.0±111.6
2-3	Loser	11	27.3±28.0	148.7±117.0	-121.0±109.2
	Stable	11	62.7±87.8	49.2±51.6	13.0±54.0
3-4	Loser	16	62.5±58.0	128.9±91.6	-66.5±97.6
	Stable	16	49.3±84.0	64.0±74.0	-15.6±64.6
4-5	Loser	10	82.9±77.0	102.0±166.0	-20.2±163.0
	Stable	10	59.1±61.6	54.8±44.8	4.3±92.6
5-6	Loser	19	59.0±91.8	118.7±56.6	-59.8±114.0
	Stable	19	79.8±55.0	72.2±43.0	6.0±58.1
1-6	Loser	36	66.7±64.8	100.0±65.0	-35.0±96.2
	Stable	36	66.3±104.0	43.0±51.0	23.6±127.0

TABLE 4.36: Mean ± Standard Deviation (SD) of gingival crevicular fluid CRP secretion (ng/minute) for matched pairs of loser and stable sites at initial visit, subsequent visit and between each pair of these successive visits (and over one year).

Visits	Site Group	Number	CRP Secretion (ng/minute) Mean ± SD Initial Visit	Subsequent Visit	Difference
1-2	Loser	17	15.2±15.6	28.1±18.4	-13.0±19.0
	Stable	17	17.1±24.0	11.2±11.6	6.8±18.1
2-3	Loser	11	17.7±21.1	51.5±34.7	-33.0±25.7
	Stable	11	22.3±36.5	17.3±24.6	4.96±31.8
3-4	Loser	16	16.9±16.2	35.7±17.6	-19.6±23.1
	Stable	16	15.5±29.6	16.7±22.0	1.8±18.7
4-5	Loser	10	30.0±38.2	34.5±18.5	-3.5±33.0
	Stable	10	21.2±24.4	20.8±10.4	0.8±24.6
5-6	Loser	19	20.4±26.9	39.4±34.8	-18.9±38.9
	Stable	19	18.1±18.7	20.7±18.9	-1.8±9.9
1-6	Loser	36	25.6±37.00	41.4±32.2	-16.0±46.2
	Stable	36	17.2±16.7	17.5±26.3	-0.3±28.0

4.6.2.2 Comparison of GCF CRP levels for matched pairs of loser and stable sites

In this study the levels of GCF CRP concentration and secretion for matched pairs of loser and stable sites within each patient were compared (loser versus stable) at the initial visit, the subsequent visit and for the differences between these visits using a paired t-test. The levels of CRP for each site group (loser and stable) at initial visit and at the subsequent visit were compared (stable versus stable, and loser versus loser) using the same statistical test. The results of the comparisons of CRP concentration and secretion are shown in Table 4.37 and Table 4.38 respectively.

For CRP concentration, no significant difference was found between loser and stable sites (loser versus stable) at each initial visits except for the successive visit 2-3 where CRP levels was significantly ($P< 0.05$) higher at stable sites than loser sites. CRP concentration was significantly ($P<0.01$) higher for loser sites than stable sites (loser versus stable) at each subsequent visit for all occasions. CRP concentration was significantly higher ($P<0.01$) for loser sites when it compared between initial and subsequent visits (loser versus loser) on all occasions, while for stable sites (stable versus stable) a significant ($P<0.05$) difference was found only for visits 1-2 and 1-6 (over one year). The difference (the response) in CRP concentration for loser sites over each pair of

successive visits (and over one year) was significantly higher ($P<0.01$) when it was compared with the response for the stable sites (difference at loser versus difference at stable sites).

For CRP secretion, no significant difference was found at each initial visit between loser and stable sites (loser versus stable sites) for most occasions except for visits 4-5 and 1-6 where CRP secretion was significantly ($P<0.05$) higher at loser sites than at stable sites. At the subsequent (recall) visit of each pair of successive visits and over one year, CRP secretion was significantly higher for loser sites than for stable sites (loser versus stable). A significant ($P<0.01$) difference was found for loser sites between the initial and subsequent visits (loser versus loser) on all occasions, while for stable sites (stable versus stable) CRP concentration was significantly different ($P<0.05$) for visits 1-2. The difference (response) in CRP secretion between the initial and the subsequent visit was significantly higher for loser sites than for stable sites (difference for loser versus difference for stable) on all occasions.

TABLE 4.37: Paired t-test analysis for CRP concentration (ng/ml) between matched pairs of loser and stable sites at the initial visit, the subsequent visit, and their difference between these successive visits (and over one year), as well as within each site group between the initial and subsequent visits.

Paired T-Test
P-Value

			Loser Versus Stable		Between Successive Visits		
Visits	Site Group	Number	Initial Visit	Subsequent Visit	Loser Versus Stable	Loser Versus Loser	Stable Versus Stable
1-2	Loser	17	> 0.05	<0.01	<0.01	<0.01	<0.05
	Stable	17					
2-3	Loser	11	<0.01	<0.01	<0.01	<0.01	> 0.05
	Stable	11					
3-4	Loser	16	> 0.05	<0.01	<0.01	<0.01	> 0.05
	Stable	16					
4-5	Loser	10	> 0.05	<0.01	<0.01	<0.05	> 0.05
	Stable	10					
5-6	Loser	19	> 0.05	<0.01	<0.01	<0.01	> 0.05
	Stable	19					
1-6	Loser	36	> 0.05	<0.01	<0.01	<0.01	> 0.05
	Stable	36					

TABLE 4.38: Paired t-test analysis for CRP secretion (ng/minute) between matched pairs of loser and stable sites at the initial visit, the subsequent visit and between these successive visits (and over one year), as well as within each site group between the initial and subsequent visits.

			Paired T-Test P-Value				
			Loser Versus Stable		Between Successive Visits		
Visits	Site Group	Number	Initial Visit	Subsequent Visit	Loser Versus Stable	Loser Versus Loser	Stable Versus Stable
1-2	Loser	17	>0.05	<0.01	<0.05	<0.01	<0.05
	Stable	17					
2-3	Loser	11	>0.05	<0.01	<0.05	<0.01	> 0.05
	Stable	11					
3-4	Loser	16	>0.05	<0.05	<0.05	<0.01	> 0.05
	Stable	16					
4-5	Loser	10	<0.05	<0.01	<0.05	<0.05	> 0.05
	Stable	10					
5-6	Loser	19	> 0.05	<0.05	<0.05	<0.01	> 0.05
	Stable	19					
1-6	Loser	36	<0.05	<0.01	<0.01	<0.01	> 0.05
	Stable	36					

4.7 Predictability of GCF CRP for periodontal disease activity in periodontal patients

The main aim for this study was to investigate the role of GCF CRP levels for both secretion and concentration in predicting periodontal breakdown measured by attachment level loss in the patient group over each pair of successive visits at two monthly intervals. The aim was to investigate its predictability for periodontal breakdown over one year. The object was to investigate the role GCF CRP as either a predictive tool for periodontal destruction or as a diagnostic aid by which it could be decided that periodontal breakdown had occurred at the periodontal site. So diagnostic predictability was also investigated.

In this study, the predictability of GCF CRP levels was investigated using all loser sites where disease activity had occurred, and all stable sites where no significant breakdown had occurred as described earlier (2.7.2).

The predictability of GCF CRP was also investigated on a more stringent basis which was a selective method. In this method a matched pair of the worst loser site and the most stable site allocated according to the method described earlier (2.7.2) was used to investigate GCF CRP predictability for periodontal disease activity.

4.7.1 Predictability of GCF CRP for periodontal disease activity using all loser and stable sites

In this study the CRP predictability for periodontal disease activity determined by attachment level loss was investigated using all the loser and stable sites in the periodontal patients in whom the attachment level changes was reported earlier (Table 4.2). This method was used on the basis that disease activity could occur at any site throughout the mouth in "random bursts".

This method would give weight to the patient and site together, as a unit of experimental analysis, for disease activity.

In this study, the GCF CRP levels at a certain threshold for both concentration and secretion was used to investigate their predictability. These thresholds (Table 4.39) were based on the data of GCF CRP concentration and secretion at the subsequent visit of each two successive visit for each occasion. For each occasion, there was a certain threshold level for positive and negative CRP tests for concentration and secretion. The threshold level for positive CRP tests was that level of GCF CRP which exceeded the mean CRP level of all the loser sites at the subsequent visit for that occasion, while the threshold for a negative CRP test was that level of CRP which was equal to or < the mean CRP level for all the loser sites at the subsequent visit. In other words a test for CRP was considered a true positive test for any site which showed a CRP secretion or

concentration level higher than the mean CRP level of all the loser sites at the subsequent visit of the two successive visits at that occasion. In the same way, a test for CRP (secretion or concentration) for any site was considered a true negative test when its CRP level was equal to or < the mean level of CRP for all the loser sites at the subsequent visits of each two successive visits for that occasion. Any stable site with CRP level exceeding the positive CRP threshold was considered as being a false positive test for CRP, while any loser site with a CRP level below the positive CRP threshold was considered as being a false negative test. The positive and negative predictive value, the sensitivity and specificity of the CRP test (for both concentration and secretion) for periodontal disease activity occurring over each two successive visits and over one year (visits 1-6), were calculated using the contingency table and equations mentioned earlier in Table 2.4, and the results are shown in Table 4.39.

As shown from the results, the positive predictive value for CRP was very low and has a range of 10.8%-40% and of 10.9%-28% over each two successive visits for both concentration and secretion respectively, while the negative predictive value for CRP was very high and has range of 94.4-97.7% and of 94.6-98.0% for CRP concentration and secretion respectively for each two successive visits. So in a high percentage of cases, a positive CRP test using concentration and secretion individually would have

misclassified a stable (inactive) site a being a loser (active) site, while in about 5% of cases a negative CRP test for both concentration and secretion individually would have misclassified an active (loser) site as being inactive (stable) site. The sensitivity of the CRP test was low and had a range of 31.2-47.6% and of 37.5-50.0% for concentration and secretion respectively for each two successive visits, while a high specificity was found for the CRP test with a range of 83.3-97.0% and of 84.6-94.0% for concentration and secretion respectively for each two successive visits.

The predictability of CRP over one year was also studied, using the threshold level for positive and negative CRP tests for both concentration and secretion, which was based on the data for CRP levels recorded for all loser sites after one year. In the same way as mentioned above, the true positive and true negative as well as the false positive and false negative test was decided, and the results are shown in Table 4.39. It was found that the positive predictive value was 60% and 57.8% for concentration and secretion respectively. A high negative predictive value was found at 83.8% and 83.4% for concentration and secretion respectively. A low sensitivity of 38.6% and 37.2% was found for concentration and secretion respectively, while a very high specificity was found at 92.5% and 92% for concentration and secretion respectively. So over one year period, a positive CRP test for both concentration and secretion would have

misclassified about 40% of stable (inactive) sites as being loser sites, while 17% of loser (active) sites would have been misclassified as being stable (inactive) sites by the negative test.

TABLE 4.39: Diagnostic predictive value of gingival crevicular fluid CRP levels for periodontal disease activity measured by attachment level loss over each two successive visits (and over one year). CRP threshold levels (ng/ml), based on the data from the subsequent visits for all the loser sites at each prescribed period, was individually used.

Visit	CRP Variable	CRP Threshold Level	Positive Predictive Value(%)	Negative Predictive Value(%)	Sensitivity (%)	Specificity (%)
1-2	*Concentration	>124.7	18.4	94.4	37.5	83.3
	**Secretion	> 37.2	18.2	94.6	41.6	84.6
2-3	Concentration	>177.9	40.0	97.3	42.8	97.0
	Secretion	> 51.0	28.0	97.5	50.0	94.0
3-4	Concentration	>127.3	13.5	96.0	31.2	89.0
	Secretion	> 35.9	12.5	96.2	37.5	86.0
4-5	Concentration	>128.1	10.8	97.7	40.0	88.7
	Secretion	> 30.6	10.9	98.0	50.0	86.0
5-6	Concentration	>109.3	20.0	95.1	47.6	84.3
	Secretion	> 37.4	19.6	94.8	43.4	85.4
1-6	Concentration	>117.2	60.0	83.8	38.6	92.5
	Secretion	> 41.1	57.8	83.4	37.2	92.0

* = concentration (ng/ml)
** = secretion (ng/minute)

4.7.2 Predictability of GCF CRP for periodontal disease activity using matched pairs of loser and stable sites within each patient

In this study the CRP predictability for periodontal disease activity as determined by attachment level loss, was investigated using matched pairs of loser and stable sites in periodontal patients of whose attachment level change was determined as reported earlier (Table 4.34). This is a selective method and was used to find if there was any difference in the predictability level of GCF CRP from that method in which all loser and stable sites were included, and also to find if the predictability level was masked by the large number of stable sites.

In this study, the GCF CRP levels at a certain thresholds for both concentration and secretion was used to investigate its predictability. These thresholds (Table 4.40) were based on the data of GCF CRP concentration and secretion related to the loser sites of matched pairs only at the subsequent visit of each pair of successive visits for each occasion. For each occasion, there was a certain threshold level for a positive and negative CRP test for concentration and secretion.

The same rules which were used for selecting CRP level thresholds, true positive and negative CRP tests and the false positive and negative tests for all the loser and stable sites (4.7.1), were used in this study for selecting the thresholds for positive and negative CRP tests.

The only exception, was that the thresholds were based on the data from the loser sites of the selected matched pairs for each prescribed period.

The positive and negative predictable values, the sensitivity and specificity of CRP test (for both concentration and secretion) for periodontal disease activity which occurred over each pair of successive visits and over one year (visits 1-6), were calculated using the contingency table and equations mentioned earlier in Table 2.4, and the results are shown in Table 4.40.

As is shown from the results, the positive predictive value for CRP concentration was reasonably high on most occasions. The positive predictive value ranged from 62.5% at visits 3-4 to the highest level (100%) at visits 2-3. The negative predictive value for CRP concentration was low and ranged from 53% at visits 4-5 to the highest level of 63.5% at visits 1-6. The sensitivity for CRP concentration was also low and ranged from 31.2% at visits 3-4 to the highest level (50%) at visits 4-5. The specificity was very high for all occasions and ranged from 80% at visits 4-5 to 100% at visits 2-3.

For CRP secretion, the positive predictive value was high on all occasions and ranged from 70% to 100%, while the negative predictive value was low and ranged from 50 to 62.5%. The sensitivity was low on all occasions and ranged from 31.5 to 50%, while the specificity was high on all occasion and was ranged from 81.3 to 100%. So a positive CRP test using concentration and secretion would have

misclassified a stable (inactive) site a being a loser (active) site in between 25 to 38% of cases for CRP concentration and in between 15 to 30% of cases for CRP secretion on most occasions. A negative CRP test would have misclassified an active (loser) site as being inactive (stable) site in 38-47% of cases for CRP concentration and in between 38-42% of cases for CRP secretion.

TABLE 4.40: Diagnostic predictive value of gingival crevicular fluid CRP levels for periodontal disease activity as measured by attachment level loss over each two successive visits (and over one year). CRP threshold levels, based on the data from the subsequent visits for matched numbers of loser sites and stable sites within each patient at each prescribed period, were individually used.

Visit	CRP Variable	No. of Sites	CRP Threshold Level	Positive Predictive Value(%)	Negative Predictive Value(%)	Sensitivity (%)	Specicity (%)
1-2	Concentration	17	>118.8	75.0	58.0	37.5	87.5
	Secretion	17	> 28.1	85.7	60.0	37.5	93.0
2-3	Concentration	11	>148.7	100.0	61.1	36.0	100.0
	Secretion	11	> 51.5	83.3	62.5	45.5	90.0
3-4	Concentration	16	>128.0	62.5	54.0	31.2	81.2
	Secretion	16	> 35.7	70.0	59.0	43.7	81.3
4-5	Concentration	10	>102.0	71.0	53.0	50.0	80.0
	Secretion	10	> 34.5	100.0	50.0	50.0	100.0
5-6	Concentration	19	>118.7	81.8	62.9	47.3	89.7
	Secretion	19	> 39.4	75.0	56.6	31.5	89.5
1-6	Concentration	36	>100.0	85.0	63.5	47.2	91.6
	Secretion	36	> 41.4	85.7	58.6	50.0	94.0
All Visits	Concentration	73	>123.2	70.0	59.3	38.4	83.6
	Secretion	73	> 37.0	74.4	60.1	43.8	85.0

4.8 The ability of clinical parameters, GCF CRP levels, and microbiological variables individually or collectively to discriminate between loser and stable sites.

The purpose of this study was to find if any of the clinical parameters (PI, BOP, GI, PD), microbiological variables (motile rods and spirochaetes), GCF volume and CRP levels (concentration and secretion), alone or in combination would be able to discriminate between loser and stable sites over each pair of successive visits and over one year. The values of these variables recorded at the initial visit of each pair of successive visits, and for the whole study period of one year alone and in combination were subject to logistic discriminant analysis. The results are shown in Table 4.41, Table 4.42 and Table 4.43.

As is shown in Table 4.41, none of PI, GI, BOP, PD, motile rods or spirochaetes individually or in combination with each other could discriminate between loser and stable sites for each pair of successive visits. While GCF volume significantly ($P<0.05$) discriminated between loser and stable sites only for visits 1-2 and 2-3 when it was used alone and when used in combination with BOP.

For visits 1-6 (over one year period) as it was shown in Table 4.41, GCF volume, BOP, PD, and spirochaetes individually successfully discriminated between loser and stable sites (significance level at $P<0.05$). When BOP was used in combination (two factors) with GCF volume, with

motile rods, and with spirochaetes, it retained its ability to significantly discriminate between loser and stable sites. It was also found that both BOP and pocket depth (two factors) significantly discriminated between loser and stable sites when they were used in combination for visits 1-6 (over one year period).

In the other study, GCF CRP secretion and concentration failed to discriminate between loser and stable sites for each pair of successive visits when they were used individually but they did so for visits 1-6 (over one year period) (Table 4.42,4.43).

When CRP secretion, GCF volume, PD, BOP, GI, motile rods and spirochaetes were tested in combination for each pair of successive visits, CRP secretion and GCF volume (two factors) in combination discriminated ($P<0.05$) between loser and stable sites only at visits 2-3 and both had a significant effect for discrimination when they were combined with BOP (three factors) for this pair of visits (Table 4.42).

For visits 1-6 (over one year period), as it is shown in Table 4.42, CRP secretion alone or in combination with other variables was able to significantly discriminate between the loser and stable sites. CRP secretion and pocket depth (two factors) in combination significantly discriminated between loser and stable sites. It was also found that both CRP secretion and BOP (two factors) were significant ($P<0.05$) alone for this discrimination when they were combined with each of spirochaetes, GCF volume

and GI, while both these two variables (secretion and BOP) together with PD (three factors) significantly discriminated between loser and stable sites. This last finding is the most interesting one as it is for three factors.

When CRP concentration alone or in combination with other variables (GCF volume, PD, BOP, GI, motile rods and spirochaetes) was tested for this discrimination, the following was found (Table 4.43):

CRP concentration alone or in combination with other factors failed to discriminate between loser and stable sites for each pair of successive visits, and only GCF volume had a significant effect alone when it was tested in combination with concentration and both with BOP for visits 1-2 and 2-3 only.

For the visits 1-6 (over one year period), it was found that CRP concentration significantly ($P<0.05$) discriminated between loser and stable sites when it was tested alone or in combination with other variables (GCF volume, PD, BOP, GI, motile rods and spirochaetes). CRP concentration in combination with GCF volume (two factors) and in combination with pocket depth (two factors) as well as in combination with both GCF volume and BOP (three factors), significantly ($P<0.05$), together with these factors, discriminated between loser and stable sites. CRP concentration and BOP had a significant effect for this discrimination when they were tested together (three factors) with spirochaetes and GI. On the other hand, with

the combination of three factors (GCF CRP concentration, PD and BOP), both CRP concentration and PD had a significant effect of discrimination. The finding of a three factor effect (CRP concentration, BOP and GCF volume) for visits 1-6 in discriminating loser and stable sites was an interesting one.

TABLE 4.41: The ability of gingival crevicular fluid volume (GCF), plaque index (PI), gingival index (GI), bleeding on probing (BOP), pocket depth (PD), motile rods and spirochaetes at the initial visit of each pair of successive visits (and over one year) to discriminate individually or collectively between loser and stable sites, using logistic discriminant analysis.

Discriminant Analysis Between Loser and Stable Sites
T-Value

Variable	V1-V2	V2-V3	V3-V4	V4-V5	V5-V6	V1-V6
GCF Volume	2.30*	2.60*	0.58	0.39	1.22	3.93*
P1	1.60	0.25	1.36	-1.35	-1.33	1.90
G1	0.45	0.44	-1.34	0.32	-0.17	1.97
BOP	0.72	0.19	0.56	0.37	-1.01	5.92*
PD	1.83	0.68	0.69	1.07	-0.79	6.13*
Motile Rods	1.37	-0.25	0.90	-0.49	0.27	0.88
Spirochaetes	-0.13	0.92	0.80	1.22	0.25	2.18*
BOP	0.27	-1.13	0.33	0.19	-1.71	5.03*
GCF Volume	2.16*	2.84*	0.44	0.20	1.96	0.81
BOP	0.34	0.30	0.25	0.69	-1.21	6.11*
Motile Rods	1.16	-0.33	0.70	-0.73	0.77	-1.46
BOP	0.85	-0.19	1.08	1.17	-1.24	5.73*
Spirochaetes	0.45	0.92	-1.14	-0.29	0.81	-0.42
BOP	-0.02	-0.12	0.26	-0.22	-0.68	3.52*
PD	1.69	0.66	0.47	1.02	-0.24	3.77*

v = visit
* = statistically significant at $P<0.05$

TABLE 4.42: The ability of GCF CRP secretion, bleeding on probing (BOP), gingival index (GI), pocket depth (PD), gingival crevicular fluid (GCF) volume, motile rods and spirochaetes at the initial visit of each pair of successive visits (and over one year) to discriminate collectively between loser and stable sites, using logistic discriminant analysis.

Discriminant Analysis Between Loser and Stable Sites

	T-value					
Variable	V1-V2	V2-V3	V3-V4	V4-V5	V5-V6	V1-V6
Secretion	-0.21	-0.48	0.13	0.76	-0.14	5.74*
Secretion	-0.43	-0.44	-0.20	0.95	-0.20	5.77*
Motile Rods	1.43	-0.16	0.89	0.68	0.32	-1.18
Secretion	-0.18	-0.73	0.35	0.54	-0.22	5.45*
Spirochaetes	-0.08	1.13	-0.84	1.08	0.31	0.07
Secretion	-1.70	-2.16*	-0.29	0.65	-0.97	4.66*
GCF Volume	2.89*	3.51*	0.64	-0.09	1.63	-0.63
Secretion	-0.65	-0.78	-0.09	0.39	-0.24	4.03*
Pocket Depth	1.96	0.95	0.68	0.86	-0.81	4.14*
Secretion	-0.56	-0.74	-0.19	0.76	0.39	3.55*
BOP	1.02	0.22	1.05	-0.54	-1.28	3.50*
Spirochaetes	-0.42	0.98	-1.15	1.21	0.77	-0.82
Secretion	-1.70	-2.00*	-0.40	0.61	-0.42	3.52*
BOP	0.22	-0.04	0.44	-0.01	-1.49	3.60*
GCF	2.76*	3.49*	0.51	-0.08	1.96	-1.34
Secretion	-0.70	-0.81	-0.21	0.55	0.52	3.02*
BOP	0.26	0.82	0.32	-0.43	-0.81	2.09*
Pocket Depth	1.74	0.27	0.48	0.97	-0.33	3.18*
Secretion	-0.62	-0.78	0.13	0.5	0.46	3.60*
BOP	0.78	0.46	0.86	-0.09	-1.09	3.52*
GI	0.25	0.58	-1.47	0.31	-0.06	-1.03

V - Visit
* = statistically significant at $P<0.05$

TABLE 4.43: The ability of GCF CRP concentration, bleeding on probing (BOP), gingival index (GI), pocket depth, motile rods and spirochaetes and gingival crevicular flud (GCF) volume at the initial visit of each pair of successive visits (and over one year) to disciminate collectively between loser and stables sites, using logistic discriminant analysis.

Discriminant Analysis Between Loser and Stable Sites

	T-value					
Variable	V1-V2	V2-V3	V3-V4	V4-V5	V5-V6	V1-V6
Concentration	-0.21	-1.35	0.55	0.36	-1.02	7.14*
Concentration	-0.31	-1.32	0.35	0.45	-1.04	7.17*
Motile Rods	1.39	-0.08	0.77	-0.55	0.51	-0.87
Concentration	-0.19	-1.56	0.79	0.24	-1.08	7.05*
Spirochaetes	-0.09	1.22	-0.93	1.18	0.61	1.67
Concentration	-0.44	-1.82	0.44	0.32	-1.07	6.87*
GCF Volume	2.35*	2.87*	0.47	0.35	1.33	3.93*
Concentration	-0.29	-1.67	0.37	0.14	-0.79	6.32*
Pocket Depth	-0.76	1.14	0.56	1.02	-0.43	4.98*
Concentration	-0.48	-1.58	0.32	0.34	-0.76	6.05*
BOP	0.99	0.27	0.83	-0.29	-0.90	3.25*
Spirochaetes	-0.44	0.99	-1.14	1.17	-0.93	0.40
Concentration	-0.38	-1.54	0.32	0.26	-0.54	5.95*
BOP	-0.13	-0.71	0.17	0.07	-1.43	2.17*
GCF Volume	2.13*	2.82*	0.35	0.25	1.90	2.52*
Concentration	-0.64	-1.69	0.29	0.23	0.66	5.93*
BOP	0.16	0.25	0.13	0.28	-0.51	1.56
Pocket Depth	1.75	0.94	0.45	1.02	-0.10	4.08*
Concentration	-0.49	-1.57	0.26	0.17	-0.69	6.09*
BOP	0.72	0.51	0.79	0.15	-0.66	3.61*
GI	0.16	0.55	-1.46	0.40	-0.05	0.68

V = visit
* = statistically significant at <0.05

4.9 The ability of clinical parameters, microbiologic variables and GCF CRP levels recorded at the initial visits individually or collectively to predict periodontal disease activity at the subsequent visits.

The purpose of this study was to find if any clinical parameter (PI, GI, BOP, PD and GCF volume), microbiological variables (motile rods and spirochaetes) and GCF CRP levels (CRP concentration and secretion) recorded at the initial visit of each pair of successive visits (and over one year) could predict periodontal disease activity measured by attachment loss at the recall visits on each occasion. Logistic discriminant analysis was used to investigate this predictability either individually for each variable or collectively for two or more variables on each occasion. The results are shown in Tables 4.44, 4.45 and 4.46.

The overall agreement (the percentage of sites which are diagnosed by the predictor as being loser sites when a real attachment loss had occurred, and of sites which are diagnosed as stable site when no real attachment level change has occurred), the percentages of misclassified sites (the percentage of sites which are diagnosed by the predictor as being loser sites but no real change in attachment level was occurred, and for the sites misclassified as being stable when a real change had been occurred), the sensitivity and specificity were used to describe the predictability of each variable. Whenever the sensitivity was 10% or less it was adjusted to 10% for

normalisation of the data in the tables. For the predictability on an individual basis, the following were found:

For CRP concentration for all pairs of successive visits, the overall agreement range was 43-60%, the misclassified percentage range was 40-57%, the sensitivity range was 10-13%, the specificity was very high and ranged from 93-97%. For the one year period, the predictability was better than for any pair of successive visits; it had an overall agreement of 76%, the misclassified percentage was 24%, while the sensitivity and specificity were 47% and 93% respectively.

For CRP secretion for all pairs of successive visits,the overall agreement range was 33-67%, the misclassified percentage range was 33-67%, the sensitivity was 10%, the specificity was very high and ranged from 90-97%. For the one year period, the predictability was better than for any pair of successive visits; it had an overall agreement of 71%, the misclassified percentage was 29%, while the sensitivity and specificity was 41% and 90% respectively.

For GCF volume for all pairs of successive visits, the overall agreement range was 62-72%, the misclassified percentage range was low and had range of 28-38%, the sensitivity range was 10-13%, the specificity was very high and ranged from 93-97%. For the one year period, there was an overall agreement of 68%, the misclassified percentage was 32%, while the sensitivity and specificity were 34% and 83% respectively.

For BOP for all pairs of successive visits,the overall agreement range was 47-58%, the misclassified percentage range was 42-52%, the sensitivity was 10%, the specificity was very high and ranged from 94-97%. For the one year period, the predictability was better than for any pair of successive visits; it had an overall agreement of 67%, the misclassified percentage was 33%, while the sensitivity and specificity was 38% and 92% respectively.

For PD for all pairs of successive visits, the overall agreement range was 50-59%, the misclassified percentage range was 41-50%,the sensitivity range was 10-11%, the specificity was very high and ranged from 93-97%. For the one year period, the predictability was better than for any pair of successive visits; it had an overall agreement of 58%, the misclassified percentage was 42%, while the sensitivity and specificity was 30% and 85% respectively.

No interesting results were found for spirochaetes, motile rods, PI or GI on individual basis for their predictability, as the misclassified percentages were still around 30% or more with a low sensitivity (10%). The results over the one year period were better than most of the paired successive visits for most of these four variables.

For the predictability of the above variables when they were used in combination, the followings results were found (Tables:4.45,4.46):

For CRP concentration when it was used in combination with the other variables, the predictors failed to give good

results at the initial visit for each pair of successive visits. As in most occasions the overall agreement did not exceed 77%, and the sensitivity did not exceed 25% except on one occasion, the misclassified percentage did not get below 33% except on one occasion. Over the one year period, the predictability improved particularly for concentration, BOP and spirochaetes (three factors) where the overall agreement was 74%, the sensitivity was 52% the specificity was 94%, and 26% were misclassified.

The overall agreement for CRP concentration, BOP with either of GCF volume, PD or GI (three factors) was relatively high (the range was 75-77%), with sensitivity of 46-46%, a specificity of 94-95%, and a low percentage of misclassified ranged at 23-26%. For CRP concentration with either PD, GCF volume, motile rods or spirochaetes (two factors), the overall agreement was 74-76%, the sensitivity was 47-49%,the specificity was 91-94% and the misclassified percentage was 23-26%. When CRP secretion was used in combination with other variables (Table 4.46), there was no improvement in the predictability either for two factors or for three factors from that for combining concentration with other variables for all paired successive visits and even for the one year period.

However, it seems from the results that better predictability was found for long term over one year and when there was a high percentage of loser sites. In other wards, a high percentage of stable sites could mask or reduce the predictability level.

TABLE 4.44: The predictability of GCF CRP concentration (Concen.), CRP secretion (Sec.), gingival crevicular fluid (GCF) volume, plaque index (PI), gingival index (GI), bleeding on probing (BOP), pocket depth (PD), motile rods (MR), and spirochaetes (Spiro.) at the initial visit of each pair of successive visits (and for a one year period) in predicting periodontal disease activity individually, using logistic discriminant analysis. The percentages of overall agreement (Agr.), sensitivity, specificity and misclassified for each variable are shown.

		Predictability of Variable (%)								
Visits		CRP Concen.	CRP Sec.	GCF Volume	Motile Rods	Spiro.	PI	GI	BOP	PD
V1-V2	Overall Agr.	43	33	72	74	30	47	71	58	59
	Sensitivity	10	10	13	11	11	10	10	10	10
	Specificity	94	90	95	93	89	94	93	94	95
	Misclassified	57	67	28	26	70	53	29	42	41
V2-V3	Overall Agr.	51	40	72	28	71	52	84	55	59
	Sensitivity	10	10	10	10	10	10	10	10	10
	Specificity	97	95	97	85	95	96	96	96	96
	Misclassified	49	60	28	72	29	48	16	45	41
V3-V4	Overall Agr.	60	63	65	74	33	50	22	57	52
	Sensitivity	10	10	10	10	10	10	10	10	10
	Specificity	96	96	95	95	97	95	98	96	96
	Misclassified	40	37	35	26	67	50	78	43	48
V4-V5	Overall Agr.	60	67	62	30	76	47	82	56	58
	Sensitivity	10	10	10	10	10	10	10	10	10
	Specificity	97	97	96	95	98	97	97	97	97
	Misclassified	40	33	38	70	24	53	18	44	42
V5-V6	Overall Agr.	44	44	66	71	70	48	18	47	50
	Sensitivity	10	10	10	10	10	10	10	10	10
	Specificity	93	93	93	93	92	96	94	94	93
	Misclassified	56	56	34	29	30	52	82	53	50
V1-V6	Overall Agr.	76	71	68	71	68	56	73	67	58
	Sensitivity	47	41	34	36	33	27	34	38	30
	Specificity	93	90	83	83	83	83	89	92	85
	Misclassified	24	29	32	29	32	44	27	33	42

TABLE 4.45: The predictability of GCF CRP concentration (Conc.), gingival crevicular fluid (GCF) volume, plaque index (PI), gingival index (GI), bleeding on probing (BOP), pocket depth (PD), motile rods (MR), and spirochaetes (Spiro.) at the initial visit of each pair of successive visits (and for the one year period) in predicting periodontal disease activity in combination, using logistic discriminant analysis. The percentages of overall agreement (Agr.), sensitivity, specificity and misclassified for each variable are shown.

		Predictability of Combined Variables (%)							
Visits		Conc PD	Conc Volume	Conc MR	Conc Spiro	Conc BOP Volume	Conc BOP PD	Conc BOP GI	Conc BOP Spiro
V1-V2	Overall Agr.	45	46	43	44	72	64	61	65
	Sensitivity	10	10	10	10	15	12	10	11
	Specificity	95	95	94	95	95	95	94	95
	Misclassified	55	54	57	56	28	36	39	35
V2-V3	Overall Agr.	51	52	50	50	61	77	70	74
	Sensitivity	10	12	10	10	10	11	10	10
	Specificity	97	97	93	97	97	97	97	97
	Misclassified	49	48	50	50	39	23	30	26
V3-V4	Overall Agr.	60	62	53	58	58	56	67	68
	Sensitivity	10	12	10	11	10	10	10	10
	Specificity	96	97	94	95	96	96	96	96
	Misclassified	40	48	47	42	42	44	33	32
V4-V5	Overall Agr.	60	60	52	57	52	60	67	75
	Sensitivity	10	10	11	10	10	33	60	10
	Specificity	97	97	96	95	97	97	98	97
	Misclassified	40	40	48	43	48	40	33	25
V5-V6	Overall Agr.	52	53	52	53	54	55	53	52
	Sensitivity	11	12	11	10	12	10	10	10
	Specificity	96	97	96	96	97	95	94	95
	Misclassified	48	47	48	47	46	45	47	48
V1-V6	Overall Agr.	76	77	74	75	75	77	75	74
	Sensitivity	47	49	48	48	46	48	46	52
	Specificity	93	92	91	94	95	95	94	94
	Misclassified	24	23	26	25	25	23	25	26

TABLE 4.46: The predictability of GCF secretion, gingival crevicular fluid (GCF) volume, plaque index (PI), gingival index (GI), bleeding on probing (BOP), pocket depth (PD), motile rods (MR), and spirochaetes (Spiro.) at the initial visit of each pair of successive visits (and for over the one year period) in predicting periodontal disease activity in combination, using logistic discriminant analysis. The percentages of overall agreement (Agr.), sensitivity, specificity and misclassified are shown.

Predictability of Combined Variables (%)

Visit		CRP PD	Sec. Volume	Sec. MR	Sec. Spiro	Sec. BOP PD	Sec. BOP Volume	Sec. BOP Spiro	Sec. BOP GI
V1-2	Overall Agr.	32	36	33	34	62	72	66	62
	Sensitivity	10	10	10	10	11	15	11	10
	Specificity	90	90	90	90	95	96	94	95
	Misclassified	68	64	67	64	38	28	34	38
V2-V3	Overall Agr.	40	41	40	39	54	75	64	37
	Sensitivity	10	10	10	10	10	10	10	10
	Specificity	95	95	94	94	97	97	96	97
	Misclassified	60	59	60	61	46	25	34	63
V3-V4	Overall Agr.	63	63	62	59	59	58	72	70
	Sensitivity	10	10	10	10	10	10	10	10
	Specificity	96	96	96	96	95	96	96	96
	Misclassified	37	37	48	41	41	42	28	30
V4-V5	Overall Agr.	67	67	60	59	70	61	74	71
	Sensitivity	10	10	10	10	10	10	10	10
	Specificity	97	97	95	95	97	97	98	97
	Misclassified	33	33	40	41	30	39	26	29
V5-V6	Overall Agr.	44	42	40	41	46	54	42	46
	Sensitivity	10	10	10	10	10	10	10	10
	Specificity	93	92	92	92	95	96	95	95
	Misclassified	46	58	60	59	54	46	48	54
V1-V6	Overall Agr.	78	78	73	73	73	69	67	70
	Sensitivity	50	50	50	50	44	39	40	41
	Specificity	91	91	92	93	92	90	92	92
	Misclassified	22	22	27	27	27	31	33	30

CHAPTER FIVE

DISCUSSION

5.1 Considerations on clinical and laboratory data

5.1.1 Identification of attachment level changes

It has been found that only a small proportion of periodontal sites are defined as experiencing disease activity, and several authors have suggested that a high percentage of these could be due to measurement errors (Imrey 1986, Clark et al., 1987, Cohen and Ralls 1986). Differences between duplicate attachment level measurements exceeding 3 times the standard deviation have been used as the criterion for disease activity, assuming that recorded differences were normally distributed (Haffajee et al.,1988; Haffajee et al., 1983a,b). However, Janssen et al.(1987) have warned that a normal distribution of errors associated with duplicate measurement has never been proven and other papers have reported that differences between attachment level measurements are not normally distributed (Badersten et al., 1984d, Glavind and Loe, 1967; Consollely and Best ,1988). Acceptance of the premise that a normal distribution of errors is true underestimates probing errors of reported proportions of sites that show a 2mm gain or loss of attachment and the writers cautioned that data derived from sites using this criterion only weakly corroborate the burst hypothesis.

Many periodontologists have used > or equal to 2mm loss (Muller et.al, 1987; Harley et al., 1987) or > or equal to 3 mm loss (Lindhe et al., 1983; Haffajee et al., 1983a,b)

in clinical attachment level to determine whether or not a real change has taken place.

In the present study, attachment level measurements were taken in duplicate for each site within each patient at each visit using a pressure-controlled probe and a modified stent method described earlier (2.5.4.1). This method of attachment level measurement was found to be a reproducible one in our studies in a similar way to the onlay method used by other authors (Badersten et al., 1984d ; Isidor et al., 1984).

Our modified stent method of probing attachment level measurements showed that the differences between the duplicate readings of measurements may occasionally be as much as 2mm or more. The replicated measurements in our studies resulted in five paired sequential measurements for each group during the whole year. These sequential measurements were subjected to the linear regression analysis method described earlier (2.7.1), and the direction and extent of attachment level change over the one year period was determined using a significance level of $P<0.05$.

The most important purpose of using regression analysis, was to find the sites which were significantly not changed (the "not changing" sites). These are the sites which did not change according to trends revealed by regression analysis of probing measurements taken bimonthly for each patient (or control) for the period of the year.

The average of the standard deviations of "not changing"

sites was used to find a significantly acceptable cut off point, which was three times more than the mean of the standard deviations of all these sites. This was 2mm for the patient group, and 1.5mm for the control group, and used in determining a real change in attachment level over each prescribed period at each site.

The safety threshold method which was described earlier (2.7.1) using a certain cut off point of attachment level was used to detect significant attachment level change.

This method was based on a reducing the false positive rate of attachment level measurements, by using the frequency distribution of the standard deviations of all "not changing" sites determined by regression analysis.

It was basically used to test the selected cut off point which was mentioned above. The 2mm safety threshold for the patient group was twice the standard deviations of more than 95% of "not changing" sites determined by regression anaylsis, and its occurrence was within less than 5% of the false positive rate. This means that the chance was that the false positive results for attachment level change would be less than 5%. In other words, only less than 5% of the change in attachment level using this threshold would be due to measurement error and this was considered the false positive rate (the relative portion of sites with measured change in attachment level that occurred in the absence of real change in attachment level). The safety threshold for the control group was selected at 1.5 mm which was at least twice the standard deviations of more

than 97% of "not changing" sites in this group,and this was within less than 5% of the false positive rate.

The reasons for selecting this method was that it would give reduced false positive rates, as basically the average of the standard deviations of "not changing" sites was used to find the significantly acceptable cut off point.

This method of selecting a cut off point has already been used by Goodson et al.(1982) who used the mean of the standard deviations of the repeated attachment level measurements of all "not changing" sites determined by regression analysis (at significance level of $P<0.05$). Their selected cut off point was > three times the mean of the standard deviations of those sites (2.5mm).

Our method of identifying the attachment level changes using these cut off points (1.5 and 2mm) was confirmed when tested by using the frequency distribution of the standard deviations of all the "not changing" sites. In both methods, the safety threshold was equal to 2mm for the patient group and to 1.5mm for the control group. This difference in threshold level between the patient and control group could be due to the subject variation and/or the site variation (Badersten et al.,1984d). For example the mean pocket depth in the control group was significantly less than in the patient group ($P< 0.05$). It was found that the probing measurements became deeper and more reproducible in the deep pockets than in the shallow pockets (Janssen et al., 1987).

This method of selecting safety threshold was introduced

by Lindhe et al.(1986) who used a safety threshold of more than 2mm of probing attachment level difference to identify sites with attachment loss. They selected their safety threshold on the basis of the findings by Badersten et al.(1984d), who suggested that a probing attachment loss of more than 2mm would occur on average in only 1-5% of repeated measurements. On this basis, therefore Lindhe et al.(1986) concluded that this threshold should provide a sufficient margin of safety.

They used the frequency distribution of the standard deviations of the repetitive measurements at sites which showed no significant attachment level change by regression analysis (at $P<0.05$). They selected a cut off point of > 2mm as the safety threshold, based on the chance that false positive results would be less than 5% and the frequency of the cut off point must be less than 5% of the false positive rate.

The views of Gunsolley and Best (1988); Best et al.(1990), and Lindhe et al.(1991) on attachment level measurement errors, appear to support this method.

Gunsolley and Best (1988) estimated the error rates associated with attachment level measurements, and concluded that a large portion of perceived changes in attachment level can be attributed to false positive rates. They found that with a difference in the mean of replicated measurements greater or equal to 2mm only 16% of changes were attributable to error.

Best et al.(1990) investigated the reliability of

attachment level measurement in a longitudinal trial, and found that attachment level measurements were in agreement within 2mm in more than 95% of sites. They defined a reliable criterion for change in attachment level which was having less than a 5% chance of obtaining one false positive change in attachment level for a mouth with 120 sites. Their results point out the lack of sensitivity of standard periodontal probe measurements and that 2-3mm of change appears to be the limit of sensitivity due to the high error inherited in the measuring method.

The criteria adopted by Lindhe et al.(1989b) for considering that a site has lost significant additional amounts of attachment was an increase in the probeable attachment level of > 2mm. The choice of this threshold was based on estimate of the false positive rate for attachment losses for single measurements presented by Glavind and Loe (1976) and replicate measurements by Cunsollely and Best (1988).

Although Haffajee et al.(1983a,b) suggested a criterion of 3 standard deviations of the difference of replicate measurements, which was equal to 2.5mm rounded up to 3mm, in their study to reduce false positive detection of attachment level change, this technique (3mm threshold) has been criticised by Fleiss et al.(1991) as it has been applied to various populations not having a similar precision of measurement.

It could be concluded that our selection of a safety threshold of 2mm would be acceptable to be used for

identification of attachment level in our study as it was based on a reducing the false positive rate, and would seem to be within the limit of sensitivity of attachment level measurement (2-3mm). It could be suggested, that using a cut off point of >2mm would not necessarily be appropriate for every study to identify a real attachment level change.

5.1.2 Incidence of attachment level change

Longitudinal studies of untreated periodontal patients have indicated that attachment loss occurs at relatively few sites over the observation periods (Goodson et al. , 1982; Lindhe et al., 1983; Haffajee et al., 1983a,b; Harley and Watts 1987; Muller and Flores, 1987; Okamoto et al., 1988; Papapanou, et al.1989).

In our study, the incidence of attachment level change over one year determined by the linear regression analysis method at a significance level of $P<0.05$, was shown earlier in Table 4.1. In the patient group, 15.3% of the total sites became significantly deeper in probing attachment level, while 2.7% of the sites became significantly shallower during the one year period. When a significance level of $P<0.01$ was used, 4% of sites showed significant attachment loss in the patient group, while only 2% of sites showed significant attachment gain.

The results for attachment level change over one year using regression analysis (at $P<0.01$) were similar to those

reported by Haffajee et al.(1983a) and Goodson et al.(1982). Haffajee et al.(1983a) found that 5.1% of sites showed significant loss of attachment and 2.3% of sites showed significant attachment gain (at $P< 0.01$) over one year. Goodson et al.(1982) found that sequential attachment level measurements made at monthly intervals for a year, when analyzed by regression analysis at $P<0.01$, demonstrated a significant increase in probeable attachment level in 5.7% of all sites monitored in 22 subjects.

Our results showed that at a significance level of $P<0.05$, the incidence of attachment loss (15.3%) was higher than that found by Jenkins et al.(1988) in their longitudinal studies on 11 patients with untreated chronic adult periodontitis during a one year period. Using regression analysis ($P< 0.05$), they found that 9.8% of sites showed attachment loss of equal to or > 2mm per year,and only 6% of sites studied showed gain in attachment level.

When a safety threshold method using a cut off point of equal to or > 2mm was used to determine the change in attachment level at each periodontal site in our patient group, the percentage of loser sites over each pair of successive visits ranged from 3.2-7.4%, while 22.4% of the total sites studied became losers during the one year period. The percentage of improved sites which gained equal to or > 2mm ranged from 1.9-2.8% over each pair of successive visits while it was 0.7% for all the sites studied over one year. The results over each paired successive visits are relatively small, and close to those

reported by Harley et al., 1987, who found that 3.7% of sites became deeper and 4.5% of sites became shallower by 2mm or more over a three month period in patients with untreated periodontal disease.

With regard to the incidence of attachment loss over a one year period (22%), this was a relatively higher percentage compared to previous studies. For example studies by Lindhe et al. (1983) in their longitudinal studies on Swedish patients with untreated periodontitis over a 6 year period, found that 37% of sites lost 2 mm or more, and only 0.2% of sites showed gain of > 2mm in attachment level. An observed attachment loss of equal to or > 2mm was found in 6.2% of Swedish subjects and in 9.1% of American subjects over one year. This maybe due to method they used in measuring and identifying the attachment level change.

5.1.2 Reporting of GCF CRP data

GCF volumes are usually below 1uL, and if small errors occur in the estimation of GCF volumes, it would result in disproportionally large alterations in the concentrations of GCF constituents (Lamster et al., 1988a). These errors are more likely to happen particularly in the ultra low range of GCF volumes, as losses from evaporation could easily occur both during sampling and transfer of the sample to the Periotron. It has been agreed that expression of GCF constituent data as a total amount is preferable (Lamaster et al., 1986 and 1988a; Wolff et al., 1988),

while other investigators seems to have their reservations and usually report results both as concentration and total amounts (Giannopoulou et al., 1992; Eley and Cox et al.1992). Lamster et al.(1986) concluded that reporting of GCF constituents using activity/ collected sample could be a more sensitive indicator of periodontal pathology than standard clinical parameters. He found a disassociation between clinical parameters and the data for GCF analysis of its content when reporting it as concentration. Lamster et al. (1989) concluded that the expression of the data as total activity per sample or concentration provided a very different relationship to periodontal disease activity.
Since the GCF volume in any disturbed crevicular location is generally less than 1 uL, even small errors in the measurement of sample volume or constituent content can be grossly magnified during subsequent calculation. So using the second approach of reporting GCF CRP analysis as a total CRP amount in crevicular location/GCF sample becomes more meaningful.
In our studies, GCF CRP analysis was carried on the basis of data reported using both the concentration (the amount of CRP per unit of volume),and the total CRP amount in the sample (activity/sample). The first approach assumes that the results obtained from a small sample of the fluid will be representive of the total fluid volume. Since the volume of GCF at any site is both small and extremely variable, it is not possible to collect a standard volume of GCF from every site. Calculation of the concentration of the

constituents in GCF could be complicated by site as, when converted, it would have a GCF volume equal to or < 0 uL. Thus, sites with converted volumes of equal to or < 0 uL would not be considered in the calculation of concentration , which makes evaluation of the concentration data difficult. All sites were considered when mean GCF volume or total CRP activity were calculated. For GCF volume, samples which converted to equal or < 0 were adjusted to 0 uL.

However, in our studies on GCF CRP, it was found that there was no improvement in the associations of attachment level change or other clinical parameters with the GCF CRP when the data was analysed on the basis of either concentration and secretion. Not, even the logarithmic transformation of concentration or secretion (data not shown) made any improvement in these associations.

It could be concluded that reporting the GCF constituent data by either concentration or secretion or both may depend on the constituent to be studied.

5.2 Longitudinal study of healthy controls and chronic periodontitis patients on maintenance therapy

5.2.1 Clinical, microbiological and laboratory findings using the subject as an experimental unit of analysis

The clinical findings in the patient and control groups using the subject as an experimental unit of analysis, demonstrated that the patient group had periodontal disease as they had pocket depths > 3.5mm (Table 4.4) and a low to moderate frequency of BOP (Table 4.13) and also the data reflected that they were on maintenance therapy throughout the study period as no significant change in their pocket depth was found over each pair of successive visits and over one year. In contrast, the clinical findings demonstrated that the control group were did not have periodontal disease or gingivitis as they had a shallow pocket depth over the study period (Table 4.4) and a very low frequency of bleeding on probing throughout the study period (Table 4.14). On the other hand no change in the attachment level was reported over the study period in this group.

The association of clinical parameters (PI, GI, BOP, PD) and GCF volume with attachment level change over each pair of successive visits and over one year was studied in both groups using the subject as an experimental unit of analysis. It was found that both PI and GI had no association with attachment level change in both groups on all occasions, while BOP was significantly associated with attachment level change in the patient group on a few occasions (Table 4.7). Both pocket depth and GCF volume had no significant correlation with attachment level change in both patient and control groups except on one occasion for the patient group.

The microbiological data including the percentages of each of coccoid cells, other, motile rods and spirochaetes failed to demonstrate any association with attachment level change in both patient and control groups on any occasion (Table 4.8).

It could be concluded that the above clinical parameters and microbiological variables had no association with periodontal disease activity on subject basis. So we are not able to determine the subjects at risk of periodontal disease activity using these clinical parameters.

The studies on serum and salivary CRP levels in both patient and control groups demonstrated no association with attachment level change on any occasion (Table 4.12),and thus none of these variables could be used to diagnose subjects with periodontal disease nor determine subjects at risk of periodontal disease.

For serum CRP, as it was found to be within normal limits for the patient group, this may indicate that periodontal disease activity is not generalised enough to produce systemic changes in serum CRP.

A significantly higher salivary CRP was found in the patient group compared to the control group, and this may be due to the presence of periodontal disease within the patient group. A speculation on that increased level of salivary CRP in the patient group suggest it could be due to the GCF source, and this should not be excluded. The level of salivary CRP was found to be 1:1000 of GCF CRP, and this may support this speculation. No significant

association was found between serum and salivary CRP on most occasions, and thus the direct contribution of serum to salivary CRP may be minimal. However, further studies using salivary samples from parotid, submandibular or sublingual glands or mucosal saliva, are required to investigate its source. The previous studies (Adam and Christids, 1962; Boucher et al., 1967; and Iwamoto et al., 1979) on salivary CRP are not reliable as they used semiquantitative methods to measure its level, although no study mentioned its arbitrary level. These studies confirmed the presence of CRP in whole saliva, and showed that its incidence in the whole saliva was higher in patients with periodontal disease than in healthy control subjects, as well as they showed its absence in saliva sampled from the parotid gland.

5.2.2 Clinical, microbiological and laboratory findings using the site as an experimental unit of analysis

The associations of periodontal disease activity measured by attachment level loss with clinical, microbiological and laboratory findings was also investigated using the site as a unit of experimental analysis. The periodontal sites either collectively or in groups were studied. These included loser sites, stable sites and improved sites. The

most interesting is the studies on either of the loser and stable site groups, and the differences in their clinical, microbiological and GCF CRP levels.

This investigation was mainly carried out because the disease activity occurred at loser sites as assessed retrospectively while no significant attachment level changes was recorded for the stable sites. It was also hoped to find if each of the variables could discriminate between disease presence (at loser site) and disease absence (at stable site).

5.2.2.1 Clinical findings

The clinical parameters including PI, GI, BOP, PD and GCF volume for the each site group in the periodontal patients, and their association with attachment level change was studied for each pair of successive visits and over the one year period (visits 1-6).

The results as reported earlier (Tables 4.13-20) showed that all the clinical parameters including PI, BOP, PD, and GCF volume had no significant association with attachment level change for each pair of successive visits and over one year at each site group on most occasions.

For GI, attachment level change was found to be associated with low GI scores at each site group, and this may indicate that marginal gingivitis was well controlled throughout the study as the patient group were receiving maintenance therapy.

BOP showed no significant association with attachment level change on most occasions except for the stable site group for visits 2-3, and for loser sites over the one year period.

Our results on attachment loss association with these clinical parameters were simliar to the previous reports (Listgarten and Levin, 1981; Haffajee et al., 1983b; Badersten et al., 1985c and 1987; Fine and Mandel 1986). These studies were failed to report any significant relation between the subsequent significant loss of probing attachment level used as an indication of disease activity, and these clinical parameters. Our studies also agreed with the results of Goodson (1986), Haffajee et al.(1988), and Lindhe et al.(1989a). Goodson 1986 found that GI, BOP , PI, exhibited no association with episodic attachment loss. Haffajee et al.(1988) found that the severity of gingival inflammation measured by GI, BOP, and PI related poorly to mean attachment loss. Okamoto et al.(1988); Yoneyama et al.(1988); and Lindhe et al.(1989a) found no significant relationship between changes in probing depth and probing attachment level.

Our findings that BOP showed an association with attachment level change, specifically attachment loss at the loser sites suggests this parameter may be useful to monitor the periodontal disease during maintenance therapy over one year period, or maybe of diagnostic help for periodontal disease activity particularly if long term studies were carried out. However, although there was an association

with BOP over the one year period, the predictive value of this parameter was found to be low in our study as it will be discussed later on.

Generally, it could be concluded that none of these clinical parameters recorded at the initial visit of each paired successive visits and over the one year period could predict future attachment level loss when the site or subject were used as the experimental unit. In other words, none of them would be useful as a diagnostic aid for periodontal disease activity.

5.2.2.2 Microbiological findings

The association of subgingival bacteria including coccoid cells, other, motile rods and spirochaetes with attachment level change at each site group was investigated, and the results indicated that all these bacterial morphotypes failed to predict the disease activity identified by attachment loss at loser site group over any prescribed period including the one year period. So phase-contrast microscopy failed to predict periodontal disease activity and can not be useful. Our studies confirm the previous observations of Listgarten et al.(1986), Harley et al.(1987), Goodson 1986, and Macfarlen et al.(1988). These investigators found that dark field microscopy of subgingival bacterial counts exhibited no association with episodic attachment loss.

5.2.2.3 Laboratory findings

It was mentioned earlier (3.3.3.1), that CRP was present in GCF from healthy and diseased periodontal sites in the patients, and healthy controls. These results support the earlier reports by Aziz et al.(1990) on CRP presence in GCF. They also support the results of studies by Sibraa et al.(1991).

In the Sibbra study, no quantitative determination of GCF CRP levels was carried out as it was only a pilot study on the presence of CRP at the diseased and healthy periodontal sites. They found no difference in the incidence of CRP presence among diseased and healthy sites. With exception of the above mentioned report, no study have ever been reported on GCF CRP. So our study in 1990 was the first report on GCF CRP, and has already been supported by the results of Sibraa et al.(1991).

The levels of GCF CRP for both concentration (ng/ml) and secretion (ng/sample or ng/minute) for all the periodontal sites in the patient and control groups were studied, and their association with clinical parameters particularly attachment level measurements as well as with microbiological variables were investigated.

As shown earlier (Table 4.20) there are higher CRP levels for both concentration and secretion for all periodontal sites in the patient group compared to the control group at each visit. This might reflect the presence of periodontal

disease in the patient group, and might be due to higher GCF volumes or deeper pockets in patient group. These two speculations were investigated and are discussed later on. When the association between the change (response) in attachment level and the change (response) of CRP levels on logarithmic transformed data for both concentration and secretion over each pair of successive visits (and over one year) for all sites in the patient and control groups was investigated, CRP response was significantly associated with attachment level change in the patient group but not in the control group. This is an interesting finding where a positive significant correlation was found between attachment level change and GCF CRP levels for both concentration and secretion. In this correlation as was illustrated earlier in figures (4.4-4.9), as probing attachment level increased at the subsequent visit of each pair of successive visits (and over one year), the CRP level increased at the same visit. It was also found that as probing attachment level decreased at the subsequent visit on each occasion, the CRP level decreased. So the deeper the probing attachment level, indicated with a large difference between the initial and subsequent visit of each pair of successive visits, became, the higher the level of CRP at the subsequent visit became. These results indicated a decisive parallel association between the change in attachment level and the CRP response.

As periodontal disease activity is determined by increasing probing attachment level, which in our study was supposed

to have occurred at the subsequent visit of each occasion, and higher CRP levels were found at this visit, It could be suggested that higher CRP levels may be associated with the occurrence of periodontal disease activity retrospectively. This is the first study carried out on GCF CRP levels and attachment level change. It could be also suggested that GCF CRP may act as a supportive diagnostic tool for attachment level measurements to determine periodontal disease activity. However, this suggestion was further studied and is discussed later on.

5.3 Differences in the clinical parameters, microbiological variables and GCF CRP levels among site groups

The studies showed earlier (4.5) that there was no significant difference between the loser and stable sites for Pl, GI, and PD recorded at the initial visits on all occasions. BOP was more frequent at loser sites than stable sites over the period of one year. GCF volume was significantly higher at loser sites on three occasions. All the subgingival bacterial morphotypes studied (coccoid cells, others, motile rods and spirochaetes) were not significantly different between loser and stable sites. CRP secretion and concentration showed no significant differences between the loser and stable sites on all occasions.

So from the above results it seems that loser and stable sites have no significant difference in their initial clinical parameters,microbiological variables and CRP levels on most occasions.

It could be suggested that these factors would seem to have no effect on the response on CRP levels in the gingival crevice. Although GCF volume was significantly different on three occasions, no significant difference was found in CRP levels between loser and stable sites at the initial visits of all occasions. So this suggests that it had no effect on CRP levels for both concentration and secretion at the initial visits in the crevice. However, this was further confirmed when GCF volume showed no significant effect on CRP levels even at subsequent visits (data was not shown).

In separate studies (data was not shown) it was found that the clinical parameters (PD and GCF volume) recorded at the subsequent visits was not significantly different between loser and stable sites on most occasions. These results also cast doubt about the effect of these two factors on the response of CRP levels over each paired successive visits. It could be suggested that these factors would not govern CRP levels in the gingival crevice.

In general the above clinical, microbiological factors have no effect on CRP levels or responses in the gingival crevice at least in the short term of two months.

The effect of GCF volume and BOP on CRP levels at the initial visit for the one year period may be limited or not significant as there was no significant difference for both

CRP levels at the same initial visits between loser and stable sites on all occasions. This means that higher volume of GCF (as for loser sites) do not necessary mean higher CRP levels than in small volumes.

It may be possible to suggest that the inflammatory condition of the gingival margin or the inflammation at the base of the pocket, which lead to increased vascular permeability and increasing GCF flow (volume), will not be enough to affect CRP response in the gingival crevice. In contrast a strong episode resulting in attachment level breakdown might have this effect. So it could be concluded that the response in GCF CRP levels was not governed by either of these factors or variables. It could also be concluded that the association between attachment level change and CRP response could not be affected by any of these variables. It could be suggested at this stage that attachment level loss may govern CRP level changes in the gingival crevicular fluid at the subsequent visit.

5.4 GCF CRP level associations with clinical parameters and microbiological variables

The studies on the associations of GCF CRP levels with clinical parameters (PI, GI, BOP, PD) recorded at the initial visits for all site groups, as mentioned earlier (4.4.2), showed a significant positive association for CRP concentration with all of these variables at stable sites on all occasions, while for loser sites only CRP

concentration was significantly associated with PD on most occasions. These associations could be due to the presence of a limited inflammatory condition at the gingival margin or at the base of the pocket resulting in a limited increase of GCF CRP concentration at the time of collecting the data, as GI and BOP represent the present status of the inflammatory process in the gingival tissue or the gingival crevice. Although the pocket depth represents the past history, like the loss of attachment level, it is possible for the inflammatory process to reoccur, particularly in deep pockets as in the case of both loser and stable sites, at any time resulting in increased gingival crevicular fluid which may affect CRP concentration. So it could be concluded that the association between CRP concentration and each of GI and BOP and PD and GCF volume may be mainly due to the limited inflammatory process occurring in deeper pockets. It could also be suggested that this inflammatory process may be not strong enough to result in attachment level loss or such limited inflammation may have occurred before or after the strong episode resulted in attachment level loss.

Most interesting was the association between CRP secretion with pocket depth for loser and stable sites, and with GCF volume for all the site groups on all occasions.

Again the association between CRP secretion and pocket depth could be incidently due to the presence of deep pocket depth which did not change on most occasions, and that may participate in increased gingival crevicular fluid

flow on all occasions. Thus, a positive correlation between secretion and GCF volume which resulted for all site groups may be due to the residual pocket depth, and /or to the presence of limited inflammatory processes resulting in increased vascular permeability and increased GCF flow. Such inflammatory processes may not be strong enough to cause attachment loss.

No significant association was found between CRP levels for both concentration and secretion measured at the initial visit of each paired successive visits and for the one year period with attachment level change over each prescribed period. These indicate that CRP levels at the initial visits may not be useful predictors of periodontal disease activity.

In general, it could be concluded that the associations of CRP secretion and concentration with the inflammatory indicators such as GI, BOP and GCF volume could be due to a limited inflammatory condition occurring at either loser or stable site groups, but usually in residual deep pockets.

As no association between CRP levels at the initial visit, was found for attachment level change. This may indicate that such inflammatory processes would either not be strong enough to produce significant increased CRP levels and attachment level change, or it could be that a strong episode occurred at any time before recording of these clinical parameters and measuring the CRP levels.

The association of CRP levels with motile rods and

spirochaetes could be due to the presence of residual deep pockets in both loser and stable sites, with the main effect on stable sites due to their large number.

5.5 GCF CRP and periodontal disease activity

The initial finding of a GCF CRP change association with attachment level change at the periodontal sites in the patient group led to further investigation for the possible role of GCF CRP in predicting or diagnosing periodontal disease activity at specific sites within an individual. No studies have been reported in the literature on the ability of GCF CRP to differentiate between periodontally active (loser) versus stable sites (inactive). Two investigations were carried out in this thesis to assess this role for GCF CRP.

In the first investigation, all the loser sites and stable sites as designated earlier (4.2) were included. In this investigation, both the patient and the site had been considered as this gave more chance for the periodontal disease to be expected at any site within any subject, than if paired sites were selected within each subject as it would be in the second investigation.

The clinical data of these two site groups (all loser and all stable sites) and the their differences were already shown earlier (Tables 4.2,4.16.,4.17,4.27,4.28), and discussed earlier (5.2.2.1). A highly significant difference in attachment level change was found between

these groups on all occasions.

Three studies, during the first investigation, were conducted to investigate this possible role of GCF CRP.

The first study was carried out to investigate the site effect on CRP levels. The difference in GCF CRP levels between loser and stable site groups at the initial visit and at the subsequent visit of each pair of successive visits (and over one year) was compared (loser versus stable at the same visit).

In the second study, the appointment (visit) effect on CRP levels was investigated. CRP level for each of loser and stable site group at the initial visit was compared with corresponding level at the subsequent visit (loser versus loser or stable versus stable) between initial and subsequent visits.

In a third study, the effect of site by appointment (visit) on CRP levels was investigated. The differences in CRP levels over each pair of successive visits, were compared between loser and stable site groups.

As mentioned earlier about the hypotheses with regard to the GCF CRP association with attachment level change and periodontal disease activity (2.7), the results of the above studies on GCF CRP were shown earlier (Tables 4.30-33), and the possible CRP role in the diagnosis of periodontal disease is discussed as fellows:

The results of the first study demonstrated no significant difference in CRP levels (concentration and secretion) between loser and stable sites (loser versus stable) at the

initial visit of each pair of successive visits (and over one year). This might suggest that it is more likely that GCF CRP at the initial visit may be at its normal level at those sites which later on were designated as loser and stable sites in the subsequent visits. Alternatively, it may be that this level (at the initial visit) had already declined from high levels during the period preceding the sampling time of GCF for the initial visit, as disease could be having a burst of disease activity at any site for short periods (days or weeks), and then the site may go in remission for long periods and stay stable. On the other hand, none of the clinical parameters (PI, GI, BOP, PD) and GCF volume showed any effect on CRP levels both for concentration and secretion (5.3). So the effect of inflammation either in the gingival margin (GI) or at the base of the pocket (BOP), which could increase vascular permeability and eventually lead to increase GCF flow (the volume), might be not strong enough to increase GCF CRP. In contrast a strong episode resulting in attachment level breakdown would have this effect. This may explain a possible mechanism of catabolism and metabolism or production of CRP within the periodontal tissues. For example, tissue destruction means cellular change in the periodontal unit, the predominant presence of lymphocytes, which are the only extrahepatic source of CRP known at the present time (Murphy et al., 1991; Kuta and Baum, 1986). It also means the initiation of all the steps involved in immune response actively within the periodontal tissues

which results in bringing most of the acute phase proteins to this area. The most likely is CRP, and an increase in its accumulation in the gingival crevice as it was already been confirmed that CRP is present in the crevice (Aziz et al.1990). It could be suggested that CRP increasingly accumulated in the area in order to participate in the defence system by expressing its various biological effects. These functions have already been mentioned in the review of literature on CRP (section 1.8). The acute inflammatory process (episode) resulting in attachment level loss would seem tohave its effect on CRP levels, and may govern its presence in the gingival crevice.

In the first study, it was also found that CRP levels for both concentration and secretion for the loser site group were significantly higher than for stable site group at the subsequent visit. So,it could be concluded that higher CRP (concentration and secretion) was associated with the presence of disease activity identified, retrospectively, at the loser site at the subsequent visit. It may be suggested that the stability of the periodontal site would be associated with low levels of GCF CRP or at least no dramatic change would occur in GCF CRP during the period of remission at specific periodontal site. So the results of the first study indicated that the site had an effect on GCF CRP levels, as CRP levels are significantly higher at loser sites (where disease activity was retrospectively recorded) than at stable site (where no significant attachment loss was recorded).

In the second study, the results showed that both CRP concentration and secretion for the loser site group at the subsequent visit were significantly higher than their corresponding levels at the initial visit on all occasions (loser versus loser between visits). While for the stable site group, a significant difference was found only at the subsequent visit of the successive visits 2-3 and one year period for concentration, and only for successive visits 2-3 for secretion. So it is obvious that a dramatic change in CRP levels was occurring at recall visits for each pair of successive visits (and over one year) for loser sites where the periodontal breakdown was occurring at the same time. This could lead to the conclusion that CRP levels significantly increased with the presence of disease activity. These findings support our early results (5.2.2.3) which showed that CRP levels significantly increased with increasing probing attachment level. It could be concluded that CRP levels can significantly change in response to attachment level change over a two month interval, and this explained the appointment (visit) effect on CRP levels.

In the third study, the results confirmed those of the second study, and furthermore indicated that the change in CRP levels (for both secretion and concentration) over each paired successive visits and over the one year period was significantly higher for loser sites than stable sites. This change represented the response in CRP levels according to the change in the circumstances of crevice or

attachment level apparatus and this was most likely the response to the change in attachment level as the other factors (GI,BOP,PD,and GCF volume) were found to have no effect on its levels.

The response in CRP secretion over one occasion and for two occasions for CRP concentration, was significant for stable sites. However, this response was significantly lower when compared with that of loser sites on the same occasions. In other words, we can say that the appointment (visit) by site effect for the stable site group was limited. In contrast, the appointment by site effect on CRP levels was obvious for loser sites on all occasions. So the site effect, appointment effect and site by appointment effect on CRP levels were confirmed either for the short term over each paired successive visits, or for the long term (over one year).

Thus, the conclusion can be drawn, that the dramatic response in GCF CRP levels was due to the strong inflammatory condition which occurred at the crevice and led to periodontal tissue breakdown represented by attachment level loss.

In conclusion, and on the basis of these studies using all loser and stable sites within the patient group, CRP levels for both concentration and secretion were significantly associated with periodontal disease activity occurrence either for the short term over each paired successive visits or over the long term over one year. This means that all the hypotheses proposed on CRP levels with regard to

attachment level change and in particular the periodontal breakdown measured by attachment loss (2.7.3) were significantly rejected.

It could be suggested at this stage that CRP levels, although not useful as predictors of disease activity, may be useful aids in the diagnostic predictability of periodontal breakdown at any site in periodontal patients including healthy and disease sites.

In the second investigation, similar studies were carried out but on the basis of selecting matched paired sites within each patient as described earlier (4.7.2). In this investigation, the patient variance was ignored, and a weight was given to the site only.

This was carried out to find if the CRP level effect and its predictability for periodontal disease activity were masked by the large number of stable sites included in the first investigation. This could have a more powerful predictability but at the expense of patient weight and it may not be clinically acceptable as paired sites within each individual do not properly represent all the sites. In contrast, six sites selected in the first investigation, was suggested to represent all teeth in the mouth (Ramjford et al., 1956). The matched paired sites were significantly different in their attachment level change (Table 4.34)

The levels of CRP for both concentration and secretion for the groups of paired sites and their difference at the same visits and between visits were mentioned earlier (4.6.2) and shown in Tables (4.35,4.36).

Paired t-tests were carried to test the site effect, appointment effect and site by appointment effect on CRP levels for these matched pairs of loser and stable sites. In other words, to test the hypotheses proposed earlier (2.7.1). The results have already been mentioned in Tables 4.37 and 4.38.

The results indicated that there was a site effect on CRP levels for both concentration and secretion as it was found that CRP levels were significantly higher for the loser sites when compared with that of the stable sites at the subsequent visits on all occasions, but no significant difference was found at the initial visits of each paired successive visits except on one occasion. The results also indicated that the appointment had an effect on CRP levels, as it was found that CRP levels for loser sites (and not for stable sites) at the subsequent visits were higher than at the initial visits on all occasions. Moreover,the results also indicated that there was an appointment by site effect on CRP levels, as the response (difference) in CRP levels for both secretion and concentration over each pair of successive visits and over one year was significantly higher for loser sites than for stable sites on all occasions.

The results of the second investigation,indicated that CRP levels for both secretion and concentration at the subsequent (recall) visits were significantly associated with attachment level changes which occurred over each paired successive visits and the one year period.

From the results of the second investigation it could be concluded that although CRP levels at the initial visits have no association with attachment level loss and thus could not be used as prospective predictors of periodontal breakdown, its levels at the subsequent visits may be useful as supportive diagnostic aids to attachment level measurement in determining the occurrence of disease activity retrospectively. In other words,CRP levels may act as a diagnostic indicators for periodontal disease activity retrospectively. This speculation was further investigated and is discussed later on.

So from the results of both investigations, it could be concluded that CRP levels measured at the subsequent visits for both secretion and concentration were significantly associated with periodontal disease activity occurring over each paired successive visits and over one year. It could also be concluded that CRP levels may act as a diagnostic indicator retrospectively, but not prospectively, for attachment level loss at any site.

5.6 Predictability of clinical parameters, microbiological and GCF CRP for periodontal disease activity

The ability of clinical parameters (PI,GI,BOP,PD) and GCF volume, the subgingival bacteria including motile rods and spirochaetes, and GCF CRP levels (concentration and secretion) recorded at the initial visits of each paired successive visits (and over one year period) to predict periodontal disease activity as measured by the loss of attachment level has been investigated in our studies. Two investigations were carried out to study the predictability of these parameters.

In the first investigation the ability of the above variables to discriminate between the presence of disease activity as represented by loser sites and the absence of disease activity as represented by stable sites, either individually or in combination on each occasion, was carried out. The values of each variable recorded at the initial visit of each paired successive visits and over one year were used in this investigation to find if these variable could discriminate between loser and stable sites using logistic discriminant analysis. As shown from Tables 4.41,4.42 and 4.43, all these variables failed individually or collectively to discriminate between loser and stable sites for each paired visit. The only exceptions were for GCF volume for the successive visits 1-2 and visits 2-3, either alone or with BOP or with CRP secretion or

concentration (two factors) or with BOP and CRP secretion or concentration (three factors). However, the effect of the combined factors for these two paired visits was related to GCF volume as on visits 1-2, and to GCF volume and CRP secretion on visits 2-3.

For the one year period, CRP secretion and concentration , GCF volume, PD, BOP and spirochaetes were able to discriminate significantly between loser and stable sites when they had been used individually. In combination, PD with each of BOP, CRP secretion and CRP concentration (two factors) significantly discriminated between loser and stable site. In a combination of three factors secretion, PD and BOP; the concentration, BOP and GCF volume, these factors retained their effect and all three factors significantly discriminate between loser and stable sites. Each of these factors retained their effect when used in combination with other variables (for example with GI, spirochaetes or motile rods).

It can be concluded from that all the above variables either individually or in combination would not be able to discriminate between the active and inactive periodontal sites at least for short periods of time (for example two months) and thus doubt is cast over their use as predictors of disease activity for the short term. On other hand, over a one year period, some of these predictors seemed to be able to discriminate between active and inactive periodontal sites either individually or collectively. The most useful would be BOP and PD and GCF volume as clinical

parameters, and CRP concentration and secretion as laboratory markers to discriminate between the disease presence and disease absence, and may be useful to monitor the periodontal disease over a long term period.

However, from a clinical point it seems that these variables (CRP secretion and concentration, PD and BOP and maybe GCF volume would not be useful as reliable discriminants as they failed in the short term and succeeded in the long term. In this situation, the assessment of the cause of the periodontal disease is more important in very short term (even less than two months) in order for the clinician to be able to take the necessary therapeutic or preventive measures rather than waiting for one year when the condition may be worse and this would unacceptable.

However, the predictability of the clinical parameters (PI, GI, BOP, PD, and GCF volume), microbiological variables (spirochaetes and motile rods) and GCF CRP levels (concentration and secretion) at the initial visits of each paired successive visits and over one year period, for periodontal disease activity was studied using logistic discriminant analysis. In this study,the overall agreement, sensitivity, specificity, and the misclassified percentages were used to assess this the predictability.

The objective was to find if any of these variables individually or collectively was able to predict the periodontal breakdown before its occurrence so that necessary preventive measures could be taken. In other

words, to test these variables for their ability to determine periodontal sites at high risk of periodontal breakdown.

As mentioned previously (4.9), all of these variables either individually or collectively failed to predict periodontal disease activity over each paired successive visits due to their low sensitivity and high percentage of misclassified cases. Although, an improvement appeared for most of these variables over a one year period, they are still considered unreliable predictors due to their low sensitivity and unacceptable percentage of misclassified sites which were classified as being loser (active) while they were stable sites. On other hand, The small size of the loser sites and the large size of stable sites on predictability of these parameters should not be excluded. In conclusion, none of these variables was useful in predicting attachment level loss at any site either for the short or long term.

Our results do confirm previous reports which demonstrated the inability of clinical criteria to predict periodontal disease activity (Haffajee et al., 1983b; Badersten et al., 1985c; Jenkins et al., 1989; Kaldahl et al., 1990; Baderstent et al.,1990).

Haffajee et al.(1983b) assessed the diagnostic relationship of certain clinical parameters (BOP,PI) at each 2-months time interval to future disease activity in untreated periodontal disease patients over a 2-year period, and found that none of the parameters used either individually

or in combination was useful in predicting disease activity. Badersten et al.(1985c) found that sites with probing attachment loss were more frequent as sites with high scores for bleeding on probing. They found that the predictability value of PI, GI, BOP did not exceed 30-40%, and thus cannot be used to predict periodontal disease activity. Kaldahl et al.(1990) found that gingival bleeding and PI were not prognostic factor of attachment loss. Claffey et al.(1990) investigated the diagnostic predictability of scores for plaque, bleeding, and probing depth for probing attachment loss over 3.5 years following initial periodontal therapy. They found that accumulated PI scores gave low predictability, while accumulated BOP scores gave modest predictive values for attachment loss. Badersten et al.(1990) studied the diagnostic value of clinical scores of supragingival plaque, bleeding, and probing depth to predict probing attachment loss over 5 years of observation following nonsurgical periodontal therapy. In their study, the diagnostic predictability of either accumulated plaque scores or accumulated bleeding scores reached a maximum of about 30%, while the residual probing depth equal or > 7 mm reached a predictability of around 50%.

Jenkins et al.(1988) investigated the ability of certain clinical parameters (pocket depth, PI and gingival redness) and microbiological criteria to predict periodontal breakdown during the one year period (with examination at 2-monthly intervals) in 11 subjects with untreated advanced

periodontitis. They found that neither the PI scores,the presence of gingival redness nor the pocket depth measurements could be used in a predictive capacity for attachment loss on either site or patient basis.

Our studies on the predictability of motile rods and spirochaetes for attachment level loss, confirmed previous investigations which have been conducted to evaluate the role of subgingival bacterial morphotypes particularly spirochaetes and motiles in predicting the disease activity measured by attachment level loss. Previous retrospective studies showed that spirochaetes cannot predict periodontal disease activity measured by attachment loss (Listgarten et al.,1978; Listgarten et al.,1986; Harley et al.1987; MacFarlane et al.1988). Harley et al.1987 monitored the clinical attachment levels over 12 weeks in patients with untreated periodontal disease every four weeks. Their study showed that none of PD, bleeding on probing or subgingival bacterial morphotypes, indicated which sites would lose attachment over a 12 weeks period. They concluded that retrospective darkfield examination of sites where apparent loss of 2mm or more had occurred, failed to predict the periodontal disease activity. MacFarlen et al.(1988) investigated the possible correlations between attachment level change and spirochaetes at the baseline visit,at 2 months intervals and after one year in patients with untreated periodontitis. They concluded that the quantification of spirochaetes cannot be used reliably to identify or predict disease-active sites.

5.7 Diagnostic Predictability of GCF CRP levels at the subsequent visits for periodontal disease activity

The predictability of both CRP concentration and secretion measured at the subsequent visits of each paired successive visits and over one year for the occurrence of periodontal breakdown determined by attachment level loss at the same visit (subsequent visits) was investigated by two different studies. In the first study, all the loser and stable sites determined by the safety threshold method and classified earlier (2.7.2,2.7.3,4.2), were used. While in the second method, matched pairs of loser and stable sites within each patient were used (Table 4.35).

As was discussed earlier (5.5,5.6), CRP levels measured at the initial visits would not be useful predictors of periodontal disease activity prospectively, but they may be a useful aid for diagnostic assessment of this activity. In other words, they maybe able to determine the occurrence of periodontal disease activity measured by attachment loss retrospectively. So this suggestion was investigated and the results were mentioned earlier (Tables 4.39,and 4.40). The thresholds used for these two studies, as mentioned earlier (Tables 4.39, 4.40) were based on the data of CRP levels recorded at the subsequent visits for each prescribed period. This was because CRP levels were significantly higher for loser sites at these visits compared with their levels at the initial visits of each

prescribed period. Secondly, the response for CRP levels over each paired successive visits (and over one year) for loser sites was significantly higher than those levels for stable sites on all occasions. The selection of these thresholds was also based on the findings that CRP levels were significantly associated with increasing probing attachment loss recorded at the subsequent visits, and as no significant association was found for attachment level change and CRP levels at the initial visits and due to their failure to predict periodontal disease activity at the initial visits. So this selection method of thresholds would give a powerful positive CRP test and negative CRP test as well as a powerful sensitivity and specificity.

In the first study, when all loser and stable sites were used, CRP concentration and secretion failed to predict periodontal disease activity retrospectively either for the short term (over each paired successive visits) or over the long term (one year period). Although there was an improvement in their predictability over the one year period, but this improvement was not enough to make these variables (CRP concentration and secretion) useful diagnostic aids for periodontal disease activity. This was because of the low positive predictive values for both CRP secretion and concentration, which resulted in misclassifying a reasonable number of stable sites as being loser sites on all occasions despite their high specificity.

In the second study, when matched pairs of loser and stable

sites within each patient were used, there was a limited improvement in the predictability for both CRP concentration and secretion on all occasion, despite the fact there was no significant difference in thresholds used for positive and negative CRP tests in both studies. The positive predictive value was significantly higher while the negative predictive value was significantly lower and no significant difference could be found for either sensitivity or specificity on all occasions. No significant improvement in predictability was found between the one year period and the paired successive visits.

The improvement in positive predictive value may be due to the decrease in the stable sites used in matched pairs compared to the first method.

However, using the second method, the results again indicated that both CRP concentration and secretion are not good predictors of the occurrence of disease activity recorded at the subsequent visits (retrospectively), and thus may be not useful as a diagnostic aid in determining the presence of disease activity. The sensitivity of the test was still low and the negative predictive value still not high enough, despite the high positive predictive value. So the positive test would have misclassified the stable sites as being loser sites in between 15-40% of cases on most occasions for both concentration and secretion, although it was reasonable for one occasion only. The negative test would have misclassified the loser sites as being stable in about 40% of cases on all

occasions. However, from a clinical point of view CRP concentration and secretion could still not be considered good predictors and not useful as diagnostic aids in periodontal disease activity or course.

In general, the sensitivity of CRP tests was very low demonstrating a very high rate of false negatives. The relatively higher specificity value is probably due to the low overall number of positive tests.

The reason for the low predictive value for CRP concentration and secretion could be due to the large number of stable sites involved in the study and the small number of loser sites due to the specific method of attachment level detection.

The Periodontal destruction may, however, occur during exacerbation episodes of short duration accompanied by changes in CRP levels. The duration of such episodes may be much shorter than the intervals at which attachment level measurements were taken in the present study, and therefore the disease activity episode could have occurred at any point in time during each prescribed period (two months observation period, and over one year). It may be possible that the periodontal episodes, which resulted in attachment level loss, would either have accompanied or preceded the change in CRP levels. CRP level change could have occurred in very short periods during any exacerbation of any inflammatory condition, and then the level could have returned back to normal after the healing process or when the stimulus was removed. This is more likely as CRP is an

acute phase protein whose levels in serum have been shown (Kaplan, 1982; Pepys and Baltz, 1983) to increase rapidly during severe inflammatory processes within 72 hours and rapidly return back to normal level when healing has occurred or the inflammation subsides. In this case any alterations in the local CRP levels may have been missed. In addition the relatively low number of sites demonstrating attachment level change compromises the possibility of CRP tests to predict periodontal disease activity.

In conclusion, the results indicated that CRP concentration or secretion, using either all the loser and stable sites or their matched pairs within the patients, failed to predict the periodontal disease activity measured by attachment level retrospectively and thus they would not be useful as diagnostic aid for the periodontal disease activity.

5.8 Conclusions, summary and further suggestions

The longitudinal studies on GCF CRP in the patient and control groups showed for the first time the presence of CRP in the GCF from healthy and disease sites, and this is the first report for GCF CRP.

The development of ELISA was confirmed to be a reliable and valid method for the detection and quantification of CRP levels in both GCF and saliva as well as in serum. Our studies reliably quantified CRP in both GCF and saliva. These studies did not confirm the sources of CRP in either GCF or saliva. Further investigation of the sources of both salivary CRP and GCF CRP are required. Further studies using salivary samples from parotid, submandibular or sublingual glands or mucosal gland saliva, are required to investigate its source.

The source of GCF CRP either as a leakage from the serum, or as locally produced in the periodontium, should be investigated. Recent studies reported extrahepatic production of CRP by peripheral blood lymphocytes (Murphy et al., 1991; Kuta and Baum, 1986)), and it would be of interest to investigate CRP production by the migrating lymphocytes into the periodontium, and its possible production by the resident cells of the periodontium such as fibroblasts. Moreover, immunolocalisation of CRP in gingival biopsies may provide information on CRP deposition in the gingival tissue and possible participation in the local immune response of the periodontium particularly as

an opsonin or scavenger of necrotic tissue or damaged cells during the inflammatory response. It would be of interest to investigate if GCF CRP had the same biological function in controlling the immune response and its cells particularly the polymorphs which are the important cell in the inflammatory response of the periodontium.

The studies reported the presence of a decisive parallel association between the change in attachment level and the change (response) in GCF CRP levels for both concentration and secretion. This indicates that a dramatic change in CRP levels was occurring during the periodontal breakdown at the same time. This decisive association indicates that CRP levels change are in association with the presence of periodontal disease activity or periodontal breakdown as measured by attachment loss in our studies. So the studies provided information on the response (change) of CRP in the GCF in relation to periodontal disease activity. However, studies aiming to identify mechanisms controlling CRP dynamics (metabolism and catabolism) in the crevice are required, in order to find which factors govern its production and response.

From the current studies it can be speculated that it is attachment level which influences GCF CRP level changes since it has already been shown that these factors are not affected by gingival inflammation or measure of GI, BOP, PD.and GCF volume. The studies found no significant difference in the clinical parameters (PI,GI,PD) between the loser and stable sites on all occasions and for BOP and

GCF volume on most occasions. Thus these results excluded an effect of these factors on CRP levels. It could be concluded that the response in GCF CRP levels was not governed by any of these factors or variables. It could also be concluded that the association between attachment level change and CRP response could not be affected by any of these variables. So the effect of inflammation either in the gingival margin (GI) or at the base of the pocket (BOP), which could increase vascular permeability and eventually lead to increase GCF flow (the volume), might be not strong enough to increase GCF CRP. In contrast a strong episode resulting in attachment level breakdown might have this effect. Thus, the conclusion can be drawn, that the dramatic response in GCF CRP levels was due to the strong inflammatory condition which occurred at the crevice and led to periodontal tissue breakdown represented by attachment level loss. This may suggest possible mechanisms of catabolism and metabolism or production of CRP within the periodontal tissues. For example, tissue destruction means cellular change in the periodontal unit and the predominant presence of lymphocytes, which are the only extrahepatic source of CRP known at the present time (Murphy et al., 1991). It also leads to the initiation of all the steps involved in the immune response actively within the periodontal tissues which results in bringing most of the acute phase proteins to this area. The most likely is CRP, and an increase in its accumulation in the gingival crevice already confirmed, by our studies, to be

present in GCF. It could be suggested that CRP increasingly accumulated or was produced in the crevicular area in order to participate in the defence systems by expressing its various biological effects which have already been mentioned in the review of literature on CRP (section 1.8). The acute inflammatory process (episode) resulting in attachment level loss would seem to have its effect on CRP levels. Thus, it could be suggested at this stage that attachment level loss may govern CRP level changes or its kinetics in the gingival crevice.

It would also be interesting to find if the periodontal breakdown measured by the alveolar bone loss would have any influence on CRP response in the periodontium as already previous studies have demonstrated the role of the bone resorbing factors (IL-1B, and IL-6) in regulating the synthesis of serum CRP. On the other hand, a correlation was previously found between bone destruction and high levels of serum CRP in rheumatoid arthritis patients.

The studies confirmed the presence of a decisive correlation between the attachment level change and the change (response) in CRP levels both the short and long term. However, within the limitations of this study, GCF CRP levels for both concentration or secretion failed, prospectively and retrospectively, to predict periodontal disease activity measured by attachment level. Thus it could be concluded that CRP would not be a useful predictor or diagnostic aid for periodontal disease activity, and could not considered as a marker of the disease activity.

The Periodontal destruction may, however, occur during exacerbation episodes of short duration accompanied by changes in CRP levels. The duration of such episodes may be much shorter than the intervals at which attachment level measurements were taken in the present study and, therefore, the disease activity episode could have occurred at any point in time during each prescribed period (two months observation period, and over one year). It may be possible that the periodontal episodes, which resulted in attachment level loss, would either have accompanied or preceded the change in CRP levels. CRP level change could have occurred in very short periods during exacerbation of any inflammatory condition, and then the level could have returned back to normal after the healing process or when the stimulus was removed. In this case any alterations in the local CRP levels may have been missed. This could mean that the determination of the exact levels of CRP during these episodes or disease remission might have been missed, and this may have an influence on its predictability. This is more likely as CRP is an acute phase protein and whose changes in serum have been shown to increase rapidly during severe inflammatory processes within 72 hours and rapidly return back to normal levels when healing has occurred or the inflammation subsides. In addition the relatively low number of sites demonstrating attachment level change compromises the possibility of CRP tests to predict periodontal disease activity. However, It could be suggested that a further investigation should be carried

out to monitor CRP levels within a very short period, for example daily or weekly monitoring of CRP level , as this level rapidly increases during the chronic inflammatory condition and rapidly goes back to its normal level when the inflammatory stimulus subsides.

Our studies confirmed the previous studies about the inability of the clinical parameters (PI,GI,BOP,PD, and GCF volume), and phase-contrast subgingival bacterial counts including spirochaetes and motile rods, to predict periodontal disease activity, and thus clinically, they are not useful as diagnostic aids for periodontal disease activity.

Future studies on CRP in periodontal disease could usefully concentrate on its specific relationship to periodontal disease activity. This could be further investigated by careful monitoring of GCF CRP levels over shorter periods of time, to allow more detailed analysis of relationship of CRP levels to the destruction process. If CRP levels prove to be valuable as a biochemical marker of periodontal disease activity over shorter time periods, it would provide a valuable tool for the better management of this major cause of human tooth loss.

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List of Tables:

ChAPTER ONE:

Table 1.1: Reproducibility of attachment level measurements using onlay methods:page (pp):42.

CHAPTER TWO:

Table 2.1: Incidence of attachment level change using linear regression analysis:pp:191.

Table 2.2: Mean and standard deviations of attachment level measurements for the "not changing" sites detected by regression analysis:pp:192.

Table 2.3a: Frequency distribution of standard deviations of the "not changing" sites detected by regression analysis in the patient group:pp:193.

Table 2.3b: Frequency distribution of standard deviations of the "not changing" sites detected by regression analysis in the control group:pp:194.

Table 2.4: Contingency 2x2 table for CRP diagnostic test and its characteristics:pp:202.

CHAPTER THREE:

Table 3.1: Preliminary results on CRP in GCF using elution at 1/200, and 1/600 in PBST 20:pp:223.

Table 3.2: Preliminary results on CRP in GCF using elution at 1/250, and 1/500 in PBST 20:pp:226.

Table 3.3: Detection of GCF CRP by ELISA using serial dilutions in PBST 20:pp:228.

Table 3.4: Application of ELISA technique for measuring serum CRP:pp:232.

Table 3.5: Detection of serum CRP by ELISA using serial dilutions in PBST 20:pp:233.

Table 3.6: Comparison of ELISA technique with Laser Nephelometry for measuring CRP:pp:234.

Table 3.7: Application of ELISA technique for measuring CRP in saliva:pp:236.

Table 3.8: Detection of CRP in saliva by ELISA using serial dilutions in PBST 20:pp:237.

CHAPTER FOUR:

Table 4.1: Regression analysis of attachment level measurements in the patient and control group:pp:240.

Table 4.2: Mean and standard error (SE) for all site groups in the patients using safety threshold at 2mm:pp:243.

Table 4.3: Mean and standard error (SE) for all site groups in the patients and controls using safety threshold at 1.5mm:pp:244.

Table 4.4: Mean and standard deviation (SD) of pocket depth and attachment level measurement per subject at each visit in patient and control groups:pp:248.

Table 4.5: Mean differences in attachment level measurement per subject between each successive visits and over one year in control and patient groups:pp:249.

Table 4.6: Spearman rank correlation test between attachment level change and either of pocket depth and GCF volume in both patient and control groups:pp:251.

Table 4.7: Chi-square analysis for the association of attachment level change with plaque index (PI), gingival index (GI) and bleeding on probing (BOP) in both patient and control groups:pp:253.

Table 4.8: Spearman rank correlation test between attachment level change with coccoid cells, motile rods, other, and spirochaetes in both patient and control groups:pp:255.

Table 4.9: Levels of serum CRP and saliva CRP at each visit in both patient and control groups:pp:257.

Table 4.10: Pearson correlation test between serum CRP and saliva CRP concentrations in each of the patient and control group at each visit:pp:259

Table 4.11: t-test analysis of the differences in serum CRP levels and saliva CRP levels between patient and control groups at each visit:pp:260.

Table 4.12: Regression analysis for attachment level change and the change in the saliva and serum CRP concentrations in each of the patient and control group over each pair of successive visits:pp:262.

Table 4.13: Mean and SE of the clinical data (PI,GI,BOP,PD, and attachment level measurements) of all sites in the patient group at each visit:pp:265.

Table 4.14: Mean and SE of the clinical data (PD, and attachment level measurements) of all sites in the control group at each visit:pp:266.

Table 4.15: Mean and SE of the percentages for coccoid cells, , other, motile rods, and spirochaetes for all sites in the control and patient group at each visit:pp:281.

Table 4.16: Regression analysis and Spearman correlation analysis of the association of attachment level change with pocket depth, GCF volume at loser, stable and improved sites in the patient group:pp:285.

Table 4.17: Chi-square analysis of the association of the attachment level change with PI,GI,BOP at loser, stable and improved sites in the patient group:pp:286.

Table 4.18: Regression analysis and Spearman correlation analysis of the association of attachment level change with coccoid cells and other at loser, stable and improved sites in the patient group:pp:287.

Table 4.19: Regression analysis and Spearman correlation analysis of the association of attachment level change with motile rods and spirochaetes at loser, stable and improved sites in the patient group:pp:288.

Table 4.20: Mean and SE for GCF volume, GCF CRP concentration and secretion for all the sites at each visit in the control and patient groups:pp:291.

Table 4.21: Mean and SE of GCF CRP concentration and secretion for loser, stable and improved sites at the initial visits in the patient group:pp:294.

Table 4.22: Spearman rank correlation test between GCF CRP levels and motile rods and spirochaetes for loser, stable, improved and all the sites at the initial visits in the patient group:pp:297.

Table 4.23: Fisher test analysis for the association of GCF CRP levels with PI,GI,and BOP at the initial visits for the loser, stable improved sites and all the sites in the patient group:pp:299.

Table 4.24: Spearman rank correlation test between GCF CRP levels with pocket depth and GCF volume at the initial visits for the loser, stable improved sites and all the sites in the patient group:pp:302.

Table 4.25: Regression analysis for the change in the attachment level change and GCF CRP levels over each successive visits and over one year for all sites in the patient and control groups:pp:305.

Table 4.26: Regression analysis and Spearman rank correlation analysis of the association of attachment level change with GCF CRP levels at the initial visits for the loser, stable and improved sites in the patient group:pp:313.

Table 4.27: F-test and ANOVA for the differences in GCF CRP levels, GCF volume and pocket depth at the initial visits between loser stable and improved sites in the patient group:pp:316.

Table 4.28: Chi-square analysis of the difference between loser and stable sites for their PI,GI, and BOP at the initial visits in the patient group:pp:317.

Table 4.29: F-test and ANOVA for the differences in the percentage of coccoid cells, other, motile rods and spirochaetes at the initial visits between loser stable and improved sites in the patient group:pp:318.

Table 4.30: GCF CRP concentration for loser and stable sites for the patient group at the initial visit, subsequent visit and their difference for each pair of successive visits and over one year:pp:321.

Table 4.31: GCF CRP secretion for loser and stable sites for the patient group at the initial visit, subsequent visit and their difference for each pair of successive visits and over one year:pp:322.

Table 4.32: Analysis of variance for GCF CRP concentration between the loser and stable sites at the initial visits, subsequent visits and between their differences over each pair of successive visits and over one year:pp:325.

Table 4.33: Analysis of variance (ANOVA) for GCF CRP secretion between the loser and stable sites at the initial visits, subsequent visits and between their differences over each pair of successive visits and over one year:pp:326.

Table 4.34: Mean and SD of attachment level change for matched pairs of loser and stable sites within each patient over each successive visits and over one year period:pp: 328.

Table 4.35: Mean and SD of GCF CRP concentration for matched pairs of loser and stable sites within patient at the initial visit, subsequent visit and between each pair of these successive visits, and over one year:pp:329.

Table 4.36: mean and SD of GCF CRP secretion for matched pairs of loser and stable sites within patient at the initial visit, subsequent visit and between each pair of these successive visits, and over one year:pp:330.

Table 4.37: Paired t-test analysis for CRP concentration between matched pairs of loser and stable sites at the initial visit, the subsequent visit, and their differences between these successive visits and over one year period, as well as within each group between the initial and subsequent visits:pp:333.

Table 4.38: Paired t-test analysis for CRP secretion between matched pairs of loser and stable sites at the initial visit, the subsequent visit, and their differences between these successive visits and over one year period, as well as within each group between the initial and subsequent visits:pp:334.

Table 4.39: diagnostic predictability of GCF CRP levels for periodontal disease activity over each paired successive visits (and over one year) using all the loser and stable sites of the patient group:pp:340.

Table 4.40: diagnostic predictability of GCF CRP levels for periodontal disease activity over each paired successive visits (and over one year) using matched number of loser and stable sites within each patient:pp:344.

Table 4.41: the ability of GCF volume,PI,GI,BOP,PD,motile rods and spirochaetes at the initial visits to discriminate individually or collectively between loser and stable:pp: 349.

Table 4.42: the ability of GCF CRP secretion, GCF volume, GI, BOP, PD, motile rods and spirochaetes at the initial visits to discriminate collectively between loser and stable:pp:350.

Table 4.43: the ability of GCF CRP concentration, GCF volume, GI, BOP, PD, motile rods and spirochaetes at the initial visits to discriminate collectively between loser and stable:pp:351

Table 4.44:The predictability of GCF CRP concentration ,CRP secretion, GCF volume,motile rods, spirochaetes, PI, GI, BOP, and PD at the initial visits of each pair of successive visits and over one year period in predicting periodontal disease activity individually:pp:356.

Table 4.45:The predictability of GCF CRP concentration, GCF volume, motile rods, spirochaetes,PI,GI,BOP, and PD at the initial visits of each pair of successive visits and over one year period in predicting periodontal disease activity in combination:pp:357.

Table 4.46:The predictability of GCF CRP secretion, GCF volume, motile rods, spirochaetes,PI,GI,BOP, and PD at the initial visits of each pair of successive visits and over one year period in predicting periodontal disease activity in combination:pp:358.

List of Figures:

Figure 2.1 : Modified stent method for attachment level measurement:pp:178.

Figure 3.1: Calibration curve for the Periotron 6000: pp:211.

Figure 3.2: Typical calibration curve for standard CRP sandwich ELISA:pp:220.

Figure 4.1: The frequency distribution of loser sites, stable sites and all the sites together with BOP at each visit in the patient group:pp:267.

Figure 4.2a-f: The frequency distribution of loser sites, stable sites and all the sites together,with plaque index scores at each visit in the patient group:pp:268-273.

Figure 4.3a-f: The frequency distribution of loser sites, stable sites and all the sites together, with gingival index scores at each visit in the patient group:274-279.

Figure 4.4: Regression analysis for the change in attachment level and the change in GCF CRP secretion for all the sites over the successive visits 1-2 in the patient group:pp:306.

Figure 4.5: Regression analysis for the change in attachment level and the change in GCF CRP concentration for all the sites over the successive visits 1-2 in the patient group:pp:307.

Figure 4.6: Regression analysis for the change in attachment level and the change in GCF CRP secretion for all the sites over one year period in the patient group:pp:308.

Figure 4.7: Regression analysis for the change in attachment level and the change in GCF CRP concentration for all the sites over one year period in the patient group:pp:309.

Figure 4.8: Regression analysis for the change in attachment level and the change in GCF CRP secretion for all the sites over all the visits in the patient group: pp:310.

Figure 4.9: Regression analysis for the change in attachment level and the change in GCF CRP concentration for all the sites over all the visits in the patient group: pp:311.

ABBREVIATIONS

A.a : Actinobacillus actinomyecetemcomitans
A2-M : A2-Macroglobulin
ABDC : antibody-dependent cytotoxicity
ACTH : adrenocorticotrophic hormone
ADP : adenosine diphosphate
ALM : autologous mixed lymphocyte reaction
ANOVA : analysis of variance
ATP : adenosine triphosphate
BL : B lymphocytes
BOP : bleeding on probing
BSF-2 : B-cell stimulatory factor-2
CEJ : cemento-enamel junction
C° : centigrade
CL : chemiluminescence
Con-A : concavalin A
CPS : C-polysaccharide
CRP : C-reactive protein
CTL : cytotoxic T-lymphocyte
CxRP : Cx-reactive protein
DDFM : differential dark-field microscopy
ELISA : enzyme-linked immunosorbent assay
ESR : erythrocyte sedemintation rate
FBS : fetal bovine serum
F-CRP : fast-CRP
F-test: Fisher test
GCF : gingival crevicular fluid
GI : gingival index
HRP : horse radish peroxidase
HSF : hepatocyte-stimulating factor
ICs : immune complexes
Ig : immunoglobulin
IL : interleukin
INF-B2: interferon B2
LDLP : low density lipoprotein
MCRP : membrane CRP
MIF : macrophage inhibition factor
MCF : migration chemotactic factor
NK : natural killer
OHI : oral hygiene index
OD : optic density
OPD : o-Phenylenediamine dihydrochloride.
PAF : platelet activating factor
PBMC : peripheral blood mononuclear cell
PC : phosphorylcholine
PBST : phosphate buffered saline tween
PCA : procoagulant activity
PCF : phase-contrast microscopy
PD : pocket depth
PDA : periodontal disease activity
PHA : phytohaemagglutinin
PF3 : platelet factor 3
PG : prostaglandin

P.gingivalis	:	porphomonous gingivalis
PI	:	plaque index
PMN	:	polymorphonuclear leukocytes
Pn-CPS	:	pneumococcal C-polysaccharide
PRP	:	platelet- rich plasma
rIL	:	recombinant interleukin
ROU	:	recurrent oral ulceration
SAP	:	serum amyloid P
S-CRP	:	surface CRP
SD	:	standard deviations
SE	:	standard error
TGF-B	:	transforming growth factor-B
TL	:	T-lymphocytes
TNF-a	:	tumor necrosis factor-a
m.w.	:	molecular weight
mg/l	:	milligram per litre
ng/ml	:	nanogram per millilitre
ug/uL	:	microgram/microliter